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Studies on Delayed-Type Hypersensitivity Including Attempts to Effect Transfer in Guinea Pigs with Subcellular Materials

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STUDIES ON DELAYED-TYPE HYPERSENSITIVITY, INCLUDING ATTEMPTS TO EFFECT TRANSFER IN GUINEA PIGS WITH SUBCELLULAR MATERIALS

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Errata

P. iii, l. 6 'use of' for 'use'

P. 5, l. 34 'occasional' for 'occasionsl'

P. 7, l. 16, 21 'Kirchheimer' for 'Kircheimer'

P. 9, l. 9 'considered' for 'conisdered'

P. 22, l. 30 'from them' for 'from the'

P. 35 Table 1. Cell Volume/donor in ml.

P. 43, l. 14 'effective' for 'effect in'

P. 60, l. 7 'lifetime' for 'live-time'

P. 77, l. 19 'about' for 'about'

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ABSTRACT

The initial observation that delayed-type hypersensitivity could be transferred from an allergic to a normal animal by means of living white cells was made at The Rockefeller Institute many years ago. Since that time it has been recognized that cellular transfer appears to be a unifying principle underlying both contact dermatitis and tuberculin hypersensitivity. Later studies in man have suggested that the phenomenon can be reproduced with use cell-free extracts. Similar attempts have been made with extracts of cells from sensitized guinea pigs but, there exist only three reports in the literature which claim that transfer of contact or tuberculin hypersensitivity can be readily accomplished between animals (guinea pigs) by means of disrupted cells or subcellular materials.

The importance of transfer of cell-free materials is great indeed, since it would permit an understanding of the mechanism of the phenomenon in the light of known immunological responses. The ease of accomplishing passive transfer with living cells taken from well-sensitized donors is such that the cited reports have left questions to be answered. Accordingly, the criteria that we demand of transfers made with living cells were brought to the evaluation of transfers with extracts. These criteria included (a) that the reactions of recipients be clearly above the level of toxicity of the allergen on normal guinea pigs' skin; (b) that the reactivity be shown to be specific for the inciting allergen; (c) that the same material injected into duplicate recipients give consistent results; and (d) that chemical depilation of test sites should not be mandatory for reactivity to become manifest.

As starting material, cells were obtained from donors that had been sensitized to contact reactivity with simple chemical allergens by any one of three methods (percutaneous, intradermal and mycobacterial-potentiated sensitizations). In addition, we used guinea pigs sensitized to tuberculin only. The cells used for transfer were mononuclear cells from paraffin oil-induced peritoneal exudates, lymph nodes and splenic cells. Prior to disruption, transfers were made of living cells of most pools to validate the 'competency' of the cells; indeed, high levels of transferred sensitivity resulted from the injection of a moderate amount of living cells, e.g., cells of one source obtained from one to two donors. The cell pools were then disrupted by three different methods (sonication, freezing and thawing, and

homogenization). Extracts from 2 to 22 times as many cells were transferred as were required to obtain strong transfers with living cells. Transfers were made by the intraperitoneal, intravenous or intradermal routes. The recipients, and normal animals in parallel, were subjected to contact or tuberculin testing at selected intervals to ensure that reactivity which might have arisen either quite soon or only after the passage of some time would be detected. The testing was conducted from four hours before transfer up to 12 weeks afterwards.

Sonic disruption was carried out with three different oscillators and in eight different media with the idea that additives (e.g., cysteine, RNA, serum) might offer protection for liberated factors. Of 88 recipients of sonically disrupted cells, all but one could be excluded as possible instances of transfer because they failed to satisfy the criteria cited above. The one recipient can not be excluded as representing a possible instance of transfer, although insufficient evidence was obtained to be sure, and many repetitions of the same procedure gave no clue that the result could be reproduced. Cells disrupted by repeated freezings and thawings were transferred into 19 recipients, of which there was no instance of reactivity suggesting that transfer had been accomplished. Cells were homogenized in sucrose media, and microsomal and 'cell sap' fractions were obtained therefrom;this likewise failed in 21 recipients to effect transfer of specific contact hypersensitivity.

Referring to the published reports mentioned above, not one could be confirmed in this work. The work of Jeter, Tremaine and Seebohm, from which transfer of contact hypersensitivity by means of sonically disrupted cells was inferred, appears to involve errors of interpretation. Likewise, the initial report of Turk (discounted in more recent work by Turk and Asherson) that contact hypersensitivity could be transferred with frozen and thawed cells or cell fractions of homogenates was not substantiated. The third report, that of transfer of tuberculin hypersensitivity by Cummings, Patnode and Hudgins, appears to merit little consideration.

In the above reports in which transfer of contact hypersensitivity was claimed, skin test sites had been freed of hair stubble by chemical depilatories. An investigation of the effects of chemical depilatory applied to skin sites, either prior to testing or after testing, indicated that the incidence of irritative reactions present at 24 hour readings, i.e., weak reactivity

which, uncritically, might perhaps be considered as 'positive', was about 60% greater, both to the 'specific allergen' and to a non-specific chemical allergen, when these were applied on depilated sites as compared to untreated sites. Beyond this, such irritative responses on depilated sites were found to occur with equal frequency and intensity on normal guinea pigs as on recipients of cell extracts. The irritative reactions artifactually produced by chemical depilation appear to have led the above mentioned authors to conclude that transfer had been accomplished; none of them tested for specificity of the 'reactions' to determine whether reactivity was related solely to the chemical allergen used to sensitize the donors.

Testing for specificity appeared to require use of non-related chemical allergens that are capable of combining with protein in the same way in which the specific allergen does. This need led to a preliminary testing of animals that had been actively sensitized. Animals sensitized to dinitrochlorobenzene, tested with a battery of structurally distinct allergens, failed to disclose notable cross-reactivity. In animals rendered exquisitely sensitive to picryl chloride by a special method, however, cross-reactions were seen to occur to all of the compounds tested, but only weakly, and none of these approached the level of intensity of reactions to the specific allergen. Next, recipients of competent living cells were tested with several chemical allergens. Cross-reactions of minor degree were found to occur on those animals, only, which had acquired reactivity to picryl chloride of very high intensity. From these studies, it was evident that the major component of reactivity in sensitized animals was directed to the specific chemical allergen. It could be concluded that, where the degree of specific sensitivity is not great, chemically unrelated allergens can be used justifiably for testing in parallel with the specific allergen to detect non-specific reactivity. Evidence of specificity is particularly necessary in recipients of subcellular materials, in which reactivity to the 'specific' allergen was so variable and so very weak. For this purpose, o-chlorobenzoyl chloride was chosen, and it was used at slightly irritative concentration. The results of such cross testing indicated that most recipients of cell extracts responding weakly to picryl chloride were equally reactive to the non-specific allergen, and that these recipients could, with justification, be excluded as possible instances of transfer of specific hypersensitivity.

From these several studies, we conclude that no reproducible method is available to effect transfer of either contact or tuberculin hypersensitivity between guinea pigs with non-living materials. The few reports of transfer with such materials appear to hold serious flaws. Whether the goal of effecting transfers in this way can be attained seemingly remains moot.

Curiously, the reports of transfer in man suggest not only the possibility of ready transfer with extracts ('transfer factor') but the statement is made that the transferred sensitivity is long-lasting. In contrast, observations made in guinea pigs had indicated that transferred sensitivity to tuberculin fades within 7 to 10 days. In order to explore the basis for apparently lasting transferred contact sensitivity in the guinea pig, recipients of competent cells were subjected to a single test, made at selected intervals between 1 and 87 days following transfer. Animals tested within the first week showed, as expected, high levels of passive sensitization; animals tested only at the later intervals showed either negative or very weak reactivity. Quite different was the finding on recipients that had been tested within the first three days after transfer and which showed strong positive reactions: when these animals were retested at later times (7 to 12 weeks) in parallel with previously untested recipients, only those that had experienced the early test exhibited sustained high levels of sensitivity. The results indicated strongly that passive sensitization of recipients of living cells was evanescent, possibly remaining only for the lifetime of the transferred cells, and that a second phase of more durable sensitivity was the result of active sensitization by the previous single test. The hazards of active sensitization due to testing are apparent. Because the manifestations of delayed-type hypersensitivity to contact allergens and to tuberculin are similar in man and in the guinea pig, it is to be exptected that general principles will underlie both. The fact that cellular transfer of chemical allergy in man has not been accomplished readily, unlike the results reported for the transfer of bacterial hypersensitivity, whereas both types of transfer occur in parallel in the guinea pig, suggests that the principles governing transfer in man have not been fully elucidated. The reported discrepancy between the two species with regard to the results of transfer attempts with cell extracts and observed duration of passive sensitization demands more careful experimentation if the mechanisms are to be uncovered.

In an effort to learn what metabolic functions of living cells were vital for transfer, competent cells were treated with metabolic inhibitors. Agents which killed the cells, such as mercuric chloride or chloroform-saturated buffers, abolished the capacity of the cells to effect transfer. Cells that had been exposed to Mitomycin C in relatively low concentration (which would be expected to prevent cell division but not synthesis of RNA or protein) gave nearly normal transfer when transferred intravenously into recipients that were subjected to testing within a few hours. Incubation of competent cells in higher concentrations of Mitomycin C or in Actinomycin D, in which circumstance it would be expected that RNA synthesis and/or protein synthesis would be markedly inhibited, was found to abolish the ability of the cells to effect transfer even though they appeared to be viable at the time of transfer. It was concluded that for cellular transfer to be accomplished, continued synthesis of RNA and/or protein are required by the transferred cells, and that no sufficient amount of any preformed material exists within the competent cells themselves to accomplish this.

Finally, because picrylated or dinitrophenylated erythrocyte stromata given in mycobacterial adjuvant routinely produced high levels of contact sensitization, and reports in the literature indicated that conjugated soluble proteins were less efficient for this purpose, there arose the question of the natural carrier of hapten. Conjugated bovine serum albumin emulsified in complete Freund's adjuvant and picrylated tubercle bacilli in paraffin oil were found to be relatively ineffective. A model for plasma membrane systems, myelin, when conjugated with picryl chloride and given in mycobacterial adjuvant was found to be highly effective in producing contact reactivity to picryl chloride. It is suggested that the "complete antigen" that induces hypersensitivity may involve conjugation of allergen with plasma membrane material.

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We dance round in circles and suppose,
But the secret sits in the middle, and knows.

Robert Frost

Skepticism is the chastity of the intellect;
One should not surrender it lightly to the first comer.

George Santayana

I

INTRODUCTION

I. Introduction

A. Delayed-type Hypersensitivity

The phenomena which are brought together under the term 'delayed-type hypersensitivity' are among the most complex and least understood of all immunological processes. The present level of investigation in the field is still that of studying the response of the whole organism to the antigen, with great attendant difficulties in trying to dissect the causative events from the chain of secondary physiological processes.

That the delayed-type hypersensitive phenomena are truly immunological in character is indicated by the facts that (a) contact with antigen or allergen is required for the induction of the hypersensitive state; (b) the reactions after sensitization are specific for the incitant; (c) the system now recognized as controlling the delayed-type reactions is the cells of the lymphoid series; (d) once a sensitivity has been established, it may be recalled by contact with the specific allergen, analogous to the anamnestic response of antibody formation; (e) desensitization is possible to some extent, and (f) normal animals can be rendered tolerant or unresponsive to sensitization.

The roots of delayed-type hypersensitivity are embedded in the classic age of bacteriology. The phenomenon was first observed by Robert Koch (1890) when severe local and systemic toxicity reactions were seen after a crude extract of the tubercle bacillus, Old Tuberculin, was injected into tuberculous guinea pigs or humans. The first to describe and understand the diagnostic potential of the local skin reactions arising from the injection of tuberculin subcutaneously into tuberculous patients was Epstein (1891) whose description of the reaction is now classic. The names of Escherich, Virchow, Schick, von Pirquet, Mantoux and many others have been associated with early progress in the field.

Originally, delayed-type hypersensitivity was thought to be associated with tuberculosis exclusively, but it occurs in a great many infections. Among the bacterial infections are undulant fever, typhoid fever, tularemia, glanders, whooping cough, streptococcal and pneumococcal infections, and syphilis. Viral diseases in which delayed-type reactions are seen include the classical vaccinia, lymphogranuloma venereum, mumps, measles, influenza, and herpes simplex. Similar type skin reactions appear after fungal infections

such as coccidiomycosis, histoplasmosis, sporotrichosis, blastomycosis and aspergillis infection. The contact dermatitides due to poison ivy, primula, penicillin and chemical allergies are reactions of the delayed type (see review by Raffel, 1961). Finally we may mention transplantation and tumor "immunity", in which "immunity" is actually hypersensitivity, as being manifestations of delayed-type hypersensitivity. Experimental thyroiditis and experimental allergic encephalomyelitis and an experimental nephrotoxic nephritis have been considered to represent special cases of delayed-type reactivity. Some of the so-called autoimmune diseases, also, have been viewed as representing hypersensitivity of the delayed type.

The division of immunologic responses into the delayed-type and immediate-type (antibody-or reagin-mediated) was first made explicit by Zinsser (1925). The presently recognized differences between the two types of hypersensitivity may be summarized as follows:

1. Passive transfer of delayed-type hypersensitivity from an allergic animal to a normal cannot be accomplished by serum, but can by means of living lymphoid cells, thus indicating that humoral factors such as classical antibodies and reagins are not responsible.

2. There is a delay between the contact of the sensitive individual with the allergen and the appearance of the reaction, maximal reactions generally requiring 16 to 48 hours, whereas immediate reactions appear five minutes to four hours after test.

3. Histologically, delayed-type local reactions are characterized by an early dense mononuclear cell infiltration causing gross induration, while antibody-mediated reactions are predominantly polymorphonuclear with edema and arterial necrosis.

4. Hypersensitivity reactions can occur in those tissues in which antigen can be retained in high concentration for the time required for the developmental course of the reaction.

5. The reactions are not diminished appreciably by antihistaminics, but are lessened by some adrenocortical hormones.

For present purposes, no general review of the field need be presented here since relevant material will be cited in the text as it pertains to work

being discussed. Excellent reviews of various aspects of delayed-type hypersensitivity have appeared in recent years and may be consulted.[1]

B. Passive Transfer of Delayed-type Hypersensitivity

1. Historical

Before a complex biological phenomenon can be understood, it is quite often necessary to isolate the anatomical or physiological system upon which it is based. The desire to transfer delayed-type hypersensitivity from an allergic individual to a normal is based on such a rationale. In the following presentation, emphasis will be placed on the two classic systems of delayed-type hypersensitivity, tuberculin and contact chemical reactivity.

A brief account of some of the very early attempts to transfer tuberculin hypersensitivity is given by Kircheimer and Weiser (1947). The first claim of successful transfer in the guinea pig is that of Helmholtz (1909) in which dermal reactivity to O.T. was found in recipients of 4 to 5 ml defibrinated blood from heavily infected tuberculous animals two days after transfer. Simultaneously with Helmholtz, Oskar Bail (1910) published an extraordinary paper reporting the transfer of tuberculin sensitivity by means of "Brei" of organs obtained from tuberculous guinea pigs. In the light of our present knowledge of the subject, we can see some of the principles governing transfer revealed in these experiments, which at the time were unfortunately not grasped. Bail ground non-caseous lymph nodes and spleens of tuberculous animals in saline and injected them by the intraperitoneal route into normal guinea pigs. The recipients were challenged generally 24 hours later with Old Tuberculin (0.5 ml), either intraperitoneally or intrapleurally, and systemic shock leading to death was observed in most cases. To ensure that

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[1] For general review see Lawrence (1956), Boyd (1956), Pappenheimer et al. (1959), and Raffel (1961). Delayed hypersensitivity to protein antigens is reviewed by Pappenheimer and Freund (1959) and Gell and Benacerraf (1961a). Hypersensitivity to simple chemical allergens has been reviewed by Rosenheim and Moulton (1958), Chase (1959), Eisen (1959), and Schild (1962). The histology of the delayed-type reactions has been described by Dienes and Mallory (1932), Kaplan and Dienes (1959) and by LaPorte (1934) for the tuberculin reaction, and by Fisher and Cooke (1958) and Gell (1961) for the contact system. Two comprehensive reviews of the action of specific antigens upon cells from hypersensitive animals have been presented by Waksman (1958, 1959).

what he had observed was indeed passive transfer, Bail included an elaborate series of control experiments which are today worthy of admiration and emulation. He mixed tissue brei made from normal lymph nodes and spleens with live tubercle bacilli (up to 12mg moist weight) and found no systemic reactions after transfer and injection of Old Tuberculin, thereby excluding toxicity due to the presence of tubercle bacilli in his organ brei. In addition, liver brei from a tuberculous animal was unable to transfer the reaction, indicating that this property was associated with what we now know as the lymphoid system. Further, sera from tuberculous animals were unable to effect transfer, hence Bail concluded that the reaction was not produced by a circulating antibody. Tissue from normal guinea pigs or from animals immunized with horse serum or ovalbumin were similarly unable to transfer the tuberculin shock, showing that the reaction was specific for the tuberculin system. And lastly, the amount of tissue brei injected, suggesting that what we know as dose-dependency probably obtained. Bail's interpretation was that within the local tissue sites infecting microorganisms created 'receptors' which reacted with the challenging dose of tuberculin to produce a poison, similar to anaphylactotoxin. There was a difference, however, in that the 'receptor' substances were not circulating but were localized in the infected tissue. Onaka (1910a,b) working in Wassermann's laboratory was able to repeat Bail's observations, only not as regularly as Bail. In addition, he found that water-extracts of organ brei were unable to effect the transfer. Joseph (1910) also verified Bail's observations but felt that the tissue brei's from the heavily infected animals were themselves toxic, that Bail's recipients must have been sick, and that the high test doses of O.T. might have been sufficient to kill the animals. One can appreciate the difficulties in working with highly infected animals, in being sure that death was the result of tuberculin shock and not toxicity and can understand the failure of these workers to grasp the principle that the sensitivity reaction was a property of the cells of the lymphoid system and not simply of antibody-like substances associated with local infected sites.

> These experiments stimulated much work attempting to transfer tuberculin hypersensitivity and to extract the active factors from tuberculous organs, most of which has not been confirmed. French workers (Massol *et al.*, 1913) claimed that transfer of tuberculous organ breis caused recipients to react to Old Tuberculin challenge

with slight increases in body temperature but were unable to find systemic shock or death. The earliest attempt at passive transfer of tuberculin hypersensitivity in man is the rather intriguing work of Fellner (1919)) who made Pirquet intradermal test sites ("prick" method with Old Tuberculin) on tuberculin sensitive donors and transferred scrapings from these tests subcutaneously into unreactive or poorly reactive tuberculous patients. The recipient skin sites were tested with O.T. and gave positive but variable reactions. Unfortunately, no control transfers of scrapings from non-sensitive individuals were made. In this country, McJunkin (1921) reported that he could render recipient guinea pigs sensitive to intradermal test with Old Tuberculin 7-15 days after transferring a filtrate (Berkefeld N) made from ground peritoneal exudate cells and abdominal wall of guinea pigs injected intraperitoneally with large amounts of tubercle bacilli. These results were interpreted to mean that transfer of hypersensitivity had been accomplished by means of a cell-free and bacillus-free extract, but it is not unlikely that the reactions were due to active sensitization by products of the tubercle bacilli present even in the filtrate. Lange (1924) reported cutaneous reactions in the guinea pig to be obtained by injecting filtrates of tuberculous foci. Reactions did not appear before the eighth to tenth days and since the animals were tested several times, it is likely that the positive reactions may have been due, again, to active sensitization. Curiously, filtrates from non-specific inflammatory foci produced by the injection of kieselguhr seemed to give reactions to O.T. almost as strong as those from tubercle foci. It is clear that the importance of testing for specificity in transfers cannot be overemphasized.

Zinsser and Mueller (1925) claimed to have transferred tuberculin hypersensitivity into guinea pigs by sera from rabbits injected with gigantic quantities (approx. 1g) of defatted dead tubercle bacilli. In the authors' own words, however, "the exact criteria by which such results can be regularly obtained have not yet been ascertained, our results in this respect, though definite, have been irregular and occasionsl". The critical reader of this paper will note that, of the reactions seen after testing with O.T., early readings (4 hour) were often of greater intensity than those made at 24 hours. Since delipidized tubercle bacilli were employed to immunize the donor rabbits, one would expect to find significant amounts of antibody to the carbohydrate of tubercle bacilli in the serum, and it is possible that the majority of the reactions seen at 24 hours were remnants of antibody-mediated immediate-type reactions. Opposed to this result, Freund (1926) was unable to transfer local tuberculin hpersensitivity by injection of serum from tuberculous guinea pigs into the skin of normals and then testing locally with Old Tuberculin in the manner of the Prausnitz-Kuestner test. Similarly, Hanks (1935) was unable to transfer delayed-type reactions to tuberculin or ovalbumin or horse serum by passive injection of serum intraperitoneally. The latter view was supported by the experience of many workers, perhaps best described in the papers of Dienes (1927, cf. Dienes and Schoenheit, 1926) in which it was clearly shown that the immediate or evanescent-type reactions could be transferred by serum obtained from tuberculous guinea pigs sensitized to ovalbumin, but typical strong delayed-type reactions could not be transferred by serum.

transfer, is the single most useful criterion for distinguishing a delayed-type hypersensitivity from an anaphylactic-type (antibody mediated) or an atopic-type (reagin mediated) phenomenon. The parallel nature of the transfer of contact hypersensitivity and tuberculin hypersensitivity united them as different instances of a single phenomenon, and permitted the chemical contact system to serve as a useful and sensitive model for the study of the delayed type of hypersensitivity.

The efforts to effect reproducible transfer of high levels of sensitivity had been considerable, and the zeitgeist was such that the importance of Chase's results was rapidly grasped, and many laboratories immediately confirmed the results. Haxthausen (1947) was able to transfer sensitivity to dinitrochlorobenzene in guinea pigs using agar- or aleuronate-induced peritoneal exudate cells and also thymus cells. He was unable to obtain local sensitization to chemical allergens in the guinea pig or in man. Cummings et al. (1947) repeated the transfer of tuberculin sensitivity in the guinea pig using exudate cells, as did Kircheimer and Weiser (1947) using exudate cells and spleen cells. The spleen cells were found to be less effective than the peritoneal exudate cells. Stavitsky (1948) was able to transfer tuberculin hypersensitivity using exudate cells, lymph node cells, and spleen cells and in two of six guinea pigs using whole blood. He found that the transferred sensitivity lasted only four to five days. Kircheimer et al. (1949) successfully transferred systemic tuberculin shock by transfer of peritoneal exudate cells from tuberculous (BCG-infected) animals upon challenging the recipients intraperitoneally with 2 ml O. T.

The cellular transfer technique was thus established, and variations on the theme, now familiar, commenced. Metaxas and Metaxas-Buehler (1948) observed local cutaneous hypersensitiveness when they injected 0.1 ml of a sensitive cell suspension intradermally and tested the site with 1 mg O.T. 48 hours after the bleb had disappeared. In addition, markedly indurated and even Cocarde reactions at the local sites could be elicited by the injection of O.T. intraperitoneally. Their results were controlled by the injection of cells from normal animals into the opposite flank of the recipient guinea pigs, these sites remaining negative.

Haxthausen (1951) reported transfer of contact hypersensitivity to dinitrochlorobenzene with white blood cells ("buffy coat") in the guinea pig,

but not with serum or with a saline-induced peritoneal exudate containing 80-90% polymorphonuclear leucocytes, the latter observation also noted by Kircheimer et al. (1951). Wesslén (1952) overcame formidable technical difficulties to show conclusively that tuberculin sensitivity could be transferred in the rabbit and guinea pig by transferring thoracic duct cells which were 98% lymphocytes. He too observed that tuberculin sensitivity could not be transferred with plasma. Further, Schmid et al. (1953) reported the transfer of tuberculin hypersensitivity by means of pleural exudate cells. It appears then that lymphocytes and possibly monocytes have the information necessary to transfer delayed-type hypersensitivity while polymorphonuclear leucocytes do not.

Cellular transfer of contact hypersensitivity was shown to be specific. Thus Chase (1953) reported that recipients made sensitive to o-chlorobenzoyl chloride by cellular transfer did not react to picryl chloride. Further, he showed that delayed-type hypersensitivity and antibody against picryl chloride could exist in the same guinea pigs and both types of reactions could be found in recipients of such donors' cells. Antibody was detected in the recipient's circulation by means of the sensitive passive cutaneous anaphylaxis technique (Chase, 1947), it requiring several days to become apparent.

Probably the most detailed and meticulous study of the cellular transfer of tuberculin hypersensitivity is that of Metaxas and Metaxas-Buehler (1955, 1958). These workers found that if cells are mixed with tuberculin and injected intradermally local transfer reactions may be obtained. However, transfer of cells intradermally or subcutaneously failed to sensitize a skin site remote from the injection site. In addition they confirmed Chase's observation that there is a dose dependency in the transfer reaction; the more cells injected, the more intense the reaction in the recipient. It was also found that multiple tests in recipients tend to diminish the reaction intensity.

Undoubtedly the greatest contribution of the Metaxas couple concerns the existence of a latent period after transfer during which no reaction can be elicited by testing. For example, cells transferred intraperitoneally failed to give positive reactions to tests made earlier than 12 hours after transfer. However, tuberculin tests made 24 hours before intravenous transfer became positive at four hours after transfer. From this evidence, the

authors conclude that there is no or, at best, a very short latent period before the transferred cells can act, and that four hours is too short a time for the few cells transferred to secrete an antibody-like factor into the circulation to reach a high enough overall concentration to be responsible for the tuberculin reactions. The authors postulate that the cells themselves must be involved in the reaction with tuberculin. This early time of reaction after transfer of cells strikes this writer as a very cogent argument against the notion that the cells release a circulating factor which is responsible for the local reactions. It is a piece of evidence not conisdered carefully enough by those who postulate such circulating factors (Karush and Eisen, 1962).

Chase (1960) and Bauer and Stone (1961) have transferred contact and tuberculin hypersensitivity respectively between members of an isologous strain of guinea pigs. It is interesting to note in the latter paper, that if minimal numbers of exudate cells were transferred, no sensitivity could be detected during the first week but a high sensitivity appeared during the second and third weeks after transfer, suggesting the multiplication of transferred cells and possibly some cloning of cells which carry the information for the hypersensitivity. Again, no success was achieved in attempting to transfer, using disrupted cells. There is no doubt but that the hazards of repeated testing with PPD were not taken sufficiently into account.

Numerous elaborations of the transfer of delayed-type reactivity and applications to the field of disease and especially the so-called auto-immune diseases have been made. Paterson (1960) has reported the transfer of experimental allergic encephalomyelitis in rats using as recipients animals which had been rendered immunologically tolerant of the donor cells. Stone (1961) was able to confirm this result using cell transfer between isologous guinea pigs. Felix-Davies and Waksman (1961) have transferred allergic thyroiditis between guinea pigs by lymph node and spleen cells. Crowle (1960) reported the transfer of tuberculin sensitivity in mice using thymus and lymph node cells. Pfeiffer et al. (1962) have recently reported the transfer of nephrotoxic glomerulonephritis in rats by means of white blood cells.

The first successful passive transfer of transplantation immunity as evidence by accelerated graft rejection was made by Mitchison (1954), who introduced the term "adoptive immunity". Lymph nodes from sensitized mice

when grafted into normals were able to bring about an accelerated rejection of the lymphosarcoma 6C3HED. Serum or exudate cells from these same donors were unable to effect the transfer. This work greatly served to strengthen the analogy between transplantation immunity and delayed type hypersensitivity (see reviews by Brent, (1958) and Amos (1962a,b).

Brent, Brown and Medewar (1962) have built a most exquisite set of experiments upon the above foundation. They have been able to sensitize a recipient guinea pig (R) to the homograft antigens of a donor (D) by applying a skin graft and allowing it to be rejected. If an antigen extract of (D) is injected into the sensitized (R) animal a "direct" delayed-type reaction resembling a tuberculin reaction is obtained. If lymphoid cells of the sensitized animal (R) are injected intradermally into the donor (D), and "indirect" delayed-type reaction is seen. If cells from (R) plus the antigen preparation from (D) are mixed and injected into a "neutral" animal a weak but significant delayed reaction occurs, indicating that "the cutaneous inflammatory reaction is in fact provoked by a local engagement of sensitized cells with antigen". This line of experimentation is analogous to the local transfer of tuberculin hypersensitivity (Metaxas and Metaxas-Buehler, 1955). Further, non-living cells or cell extracts were unsuccessful in eliciting specific transfer reactions.

Only a few reports on attempts at interspecies transfer of delayed-type-hypersensitivity are to be found in the literature. Systemic transfer could not be effected from guinea pig to rabbit and *vice versa* by Chase *et al*. (1955) or by Harris and Harris (1957). Likewise, Lawrence (1959) reported that Rammelkamp, Thomas and Stetson had been unable to transfer tuberculin hypersensitivity from man to guinea pig or from man to rabbits. Rosenthal *et al*. (1955), also, was unable to transfer contact hypersensitivity from man to guinea pigs.

Schlange (1955) reported that he obtained weak local transfer of tuberculin hypersensitivity by human cells transferred into guinea pigs. He used cells obtained from children by means of cantharides blister fluids, mixed the cells with Old Tuberculin and injected the mixture intradermally into guinea pigs. The reactions obtained were feeble, measuring less than 10mm. Wallace (1958) claimed to transfer to normal guinea pigs reactivity to *Mycobacterium lepraemurium* by mixing exudate cells of infected rats with lepromin. Systemic transfers were very weak, but local transfer, i.e., cells

plus test antigen injected intradermally, appeared to be successful. However, the reactions were not completely specific as reactions were also obtained by mixing the lepraemurium-sensitive cells with O.T. Later, Bacon, Dabney and Wallace (1961) reported that local transfer of tuberculin hypersensitivity by rat cells mixed with O.T., and injected intradermally into guinea pigs had been accomplished.

It seems, therefore, that for the competent cells to establish systemic hypersensitivity they require a species-specific milieu. The question of whether local deposition of cells mixed with allergen produces a useful indication of hypersensitivity remains open.

To conclude this section, brief mention of the fate of the transferred lymphoid cells should be made. The fate of transferred normal cells of the lymphocytic series has been extensively studied (see Fichtelius, 1957, 1958, 1960; Rebuck and LoGrippo, 1961). In the cellular transfer of contact hypersensitivity, deWeck and Brun (1956) reported that reactions of recipients of cells from donors sensitive to dinitrochlorobenzene showed only a mononuclear infiltration of the dermis, but the intesity of the reactions shown was very weak. Najarian and Feldman (1961) reported specific accumulation of cells from tuberculin sensitive guinea pigs labelled with tritiated thymidine at tuberculin test sites. Unfortunately, insufficient controls on the specificity of these reactions, and the unusual technique of tuberculin testing in the ear of the recipients instead of the more usual intradermal test on the flank causes the writer to regard the conclusions with some reservations. Very recently, these experiments were repeated by Kay and Rieke (1963) who by testing at different times were unable to find a parallelism between inflammation and number of labelled cells present in the skin. Sites of later (3 to 4 days after transfer) PPD tests, although inflamed, did not contain more labelled cells than the sites of relatively non-inflamed Histoplasmin control tests. In agreement with these latter observations, Hamilton and Chase (1962) transferred lymph node cells from picryl chloride sensitive guinea pigs which were labelled with tritiated thymidine and were unable to find greater accumulation at specific test sites than at untested areas of skin. In addition, labelled cells appeared in the skin with much less frequency than in the lymphoid organs, the lungs or the portal areas of the liver. These observations have been confirmed by Turk (1962). It would appear that the question of whether

transfer cells actually go to the test site and participate directly in the reaction remains unresolved.

3. Cellular transfer in Man with Living Cells

Attempts to transfer delayed-type hypersensitivity in man have been reviewed by Lawrence (1959) broadly. In this section, only those experiments which bear on the nature of the transfer of tuberculin or sensitivity to chemical allergens will be discussed. A bibliography of the different systems that have been studied, e.g., viral, fungal, transfers into anergic individuals or persons exhibiting pathological alterations, is given by Lawrence.

Following the demonstration of cellular transfer of hypersensitivity in the guinea pig by Chase, Lawrence (1949) adapted this technique to man and reported the transfer of tuberculin hypersensitivity by small amounts of peripheral white blood cells separated by fibrinogen. The cells were injected intradermally in the deltoid area of pre-tested tuberculin negative subjects. Intradermal tests (1mg O.T. or 5μg PPD) were applied both to the injection site and to a distant site as well, generally the forearm. It was found that transfer of cells equivalent to packed cell volumes of 0.1ml or greater (obtained from 60-100ml whole blood) led to positive reactions in the recipients. Lawrence summarized these results (1959): "In the first twenty-four to forty-eight hours after challenge, it is usual for only the leucocyte site to respond with the development of a positive tuberculin reaction in the human subject. The unprepared skin sites and those prepared with erythrocytes or serum usually remain negative until the fourth day after challenge (sixth day after transfer) at which time generalized sensitivity becomes manifest". Determination of the onset of 'local transfer' is beset with the difficulty that intradermal injection of leucocytes causes an inflammatory reaction itself. The finding of generalized reactions after six days may well correspond to cellular transfer in the guinea pig; whether it could perhaps reflect some component of active sensitization, as by either transfer of some antigenic materials in the cells or by the few tuberculin tests, as well as the possibility of recall of previously experienced sensitizations that have not been revealed by the screening tests must be kept in mind.

Lawrence continued this work by studies on the cellular transfer of hypersensitivity to hemolytic streptococci, working with carefully chosen test concentrations and non-reacting individuals as recipients, since

the normal adult population has had exposure to streptococci in considerable proportion and there is a high frequency of hypersensitivity, often low grade. Using 0.2 to 0.3ml of packed white blood cells, of sensitive donors, (1952) in all of fourteen transfers Lawrence found previously negative recipients to exhibit generalized sensitivity to intact streptococcal cells, streptokinase-streptodornase, or M-substance. The reactivity appeared from 6 hours after transfer of cells to five days, and no cutaneous reactions developed in the streptococcus-negative recipient when the transferred leucocytes were obtained from streptococcus-negative donors. In these instances, the duration of the transferred sensitivity seemed to decrease with time over a three month period, as contrasted with the durability of tuberculin transfers. Similar observations were reported later by Lawrence and Pappenheimer (1956) in studies on the cellular transfer of hypersensitivity to diphtheria toxoid in man.

Recently, Braunsteiner et al. (1958) have reported transfer of hypersensitivity to tuleremia antigens by use of 15 to 30 x 10^6 monocytes obtained from exquisitely sensitive donors. The monocytes were obtained from cover slip windows containing the antigen, placed over scraped skin on the donors. Recipients of the monocytes became positive to tulerin on the second to fourth day and to tuberculin on the fourth day.

Some of the difficulties in interpreting the Lawrence-type cell transfers will apply also to attempts to utilize cell extracts to effect transfer in man (I-C-1) and warrant consideration here. First hypersensitivities to bacterial antigens studied, e.g., tuberculin, streptococcal, and diphtheria toxoid, are instances of infections or immunizations to which virtually every human being has been exposed previously. Although the pre-transfer testing of a prospective recipient gives a negative result, it is not impossible that there has been a previous exposure to the allergen which has induced a sensitivity that has remained at sub-threshold level or has been lost. Indeed, some rather puzzeling results have been obtained in man (Jensen et al., 1962). Cells from donors sensitive to one or more strains of tubercle bacilli were transferred into recipients negative to all the tuberculins made from the strains used. Tests were made with PPD's made from the human, avian and Battey strains. Of 26 transfers of peripheral blood cells, recipients were positive in 16 instances at 48 hours. However, in four of the transfers, the recipients were found to give positive reactions to PPD's to which the donors had not exhibited

sensitivity, and in one of the control transfers, cells from a negative donor being transferred into a negative recipient, the recipient became positive to PPD-Battey and PPD-avian. These results cast doubt as to the status of individuals as it existed prior to transfer. Further work along these lines is sorely needed.

Attempts to passively transfer delayed-type hypersensitivity to simple chemical allergens in man have obviously aroused the interest of several laboratories owing to the desire to employ allergenic substances to which one can be sure that the possible recipients have never been exposed and, by virtue of the defined chemical nature of the compounds chosen, one can have available a series of allergens that permit in a unique way the privilege of studying specificity. Most of the early attempts to transfer contact dermititides in humans remain entirely without confirmation.

> In the older literature, claims of successful transfers, mainly with blister fluids, to such allergens as iodoform, salvarsan, dinitrochlorobenzene, and even to ammonium persulfate, are numerous (Perutz, 1927; Fuhs and Riehl, 1927; von Dishoeck and Roux, 1941). The older Urbach-Koenigstein technique, in which blisters were developed over the areas of experimental contact dermatitides and blister fluid was transferred, (see Urbach, 1943), was reutilized for dinitrochlorobenzene transfer by Ballestero and Mom (1945) but these authors felt that they could transfer even when cells were excluded from the blister fluid. Failure to repeat or confirm these claims is reported by Sulzberger (1940) and Leider and Baer (1948).

Among the more recent attempts, Epstein and Kligman (1957) reported that they were able to transfer hypersensitivity to pentadecyl catechol in seven out of nine recipients, to dinitrochlorobenzene (testing with 1% in acetone) in five of thirteen recipients, and to paranitrosodimethylaniline in five of thirteen instances by the use of much larger amounts of peripheral leucocytes (approx. 1×10^8) than had been employed by others. The report by Epstein and Kligman would indicate that the possibility of cellular transfer exists in humans, even though under these circumstances positive results were irregular. It is interesting that in a third of the recipients the sensitivity to pentadecyl catechol lasted from five to fourteen months and undoubtedly this isdue to active sensitization by the testing. Another claim of positive transfer of contact hypersensitivity in man is that of Good *et al.* (1957). Sensitivity to dinitrofluorobenzene was transferred from sensitized healthy individuals into hypoglobulinemic recipients and also from a sensitized

hypogamma-globulinemic patient to healthy recipients, using an average of 8.4×10^8 peripheral white blood cells. The recipients of the sensitive cells showed a reaction to 10% dinitrofluorobenzene in acetone-olive oil, whereas the recipient of normal cells did not. It is difficult to comment on this work because specificity was not demonstrated, and because the very high test dose employed might be expected to produce toxicity in all recipients. Suffice it to say that the necessary finesse of cellular transfer to chemical allergens in human beings merits much further investigation.

Baer and Sulzberger (1952) transferred peripheral leucocytes in the same quantity as Lawrence, from patients found sensitive to any of a variety of compounds, e.g. p-phenylenediamine, turpentine, and nickel. Of the recipients, 21 of 27 were negative, two were questionable, and four were apparantly positive; these latter four were considered by the authors to represent active sensitization. Baer, et al. (1952) attempted to amplify the above experiments and scored only three out of 66 transfer recipient as positive reactors, and again the authors suggest that these three recipients were actively sensitized and that passive transfer was not accomplished.

Harber and Baer (1961) attempted to repeat the experiment of Epstein and Kligman in the dinitrochlorobenzene system only. In place of "buffy coat" leucocytes as used by Epstein and Kligman, Harber and Baer used transfusions of whole blood (500ml containing 2×10^8 white cells) from exquisitely sensitive donors who reacted to a test concentration of 0.0001% of the allergen. Upon testing recipients with 0.1% dinitrochlorobenzene all recipients were negative. With 1% dinitrochlorobenzene, the concentration used by Epstein and Kligman, two out of seven recipients had inflammatory reactions that these authors attribute to the toxicity of the allergen at this high concentration for human testing. They concluded that the transfer attempts were negative. In a discussion following the paper, Dr. Sulzberger agreed that the test concentration of dinitrochlorobenzene used by Epstein and Kligman was toxic to some human skins and that in his estimation the weak reactions reported by Epstein and Kligman did not represent positive transfer.

Haxthausen (1952) in attempting to apply the Lawrence technique of cell transfer to the chemical allergen system was able to find only one out of sixty-six recipients who appeared positive, using dinitrochlorobenzene as

the allergen. Interestingly, he was unable to transfer tuberculin hypersensitivity by the Lawrence method in nine attempts with peripheral leucocytes. In a later paper (1953), Haxthausen attempted to effect local passive transfer using freshly obtained lymph node cells from allergic volunteers. There appeared to be a greater reaction when the allergen was injected into the site of the cell injection than when the allergen was injected into a site which received a frozen and thawed aliquot of cells. However all patch tests for systemic passive transfer were negative. A recent paper claiming transfer of hypersensitivity to procaine by means of viable cells or cell extracts (Freedman and Fish,1962) can be dismissed on the grounds that the reactions were too small to be considered by most as positive, no tests for specificity were made, no tests for the toxicity of the compound were considered and no control transfers of non-sensitive cells were reported.

In summary, then, it would appear that hypersensitivity to bacterial allergens can by transferred in man by the use of small volumes of packed peripheral leucocytes, although there needs to be much more study with transfers of living cells, since the results are not wholly unambiguous. This transferred hypersensitivity is found to persist for many months in the recipients. With regard to the transfer of hypersensitivity to simple chemical allergens, the results are even more equivocal and less reproducible. The ease with which transfer of hypersensitivity to simple chemical allergens can be effected in the guinea pig suggests that technical refinements should be sought.

C. Transfer of Delayed-type Hypersensitivity with Disrupted Cells or Sub-Cellular Materials

1. Experiments with Non-Living Cellular Material in Man

Entirely unlike the early negative results cited above when transfers were attempted between guinea pigs or rabbits with non-living cells, a report by Lawrence (1954) that disrupted cells could produce sensitivity in human recipients offered apparantly the most important clue into the mysteries of delayed-type hypersensitivity (See reviews by Lawrence, 1959, 1960). In the first report, peripheral blood leucocytes from tuberculin sensitive donors that had been lysed by distilled water or had been frozen and thawed were transferred subcutaneously or intradermally into tuberculin hegative recipients. All attempts were successful. Desoxyribonuclease (DNase) was unable

to destroy the ability of the extracts to transfer, and was thereafter employed for its usefulness, with added Mg^{++}, in making the solutions less viscous for injecting. In the first paper, the time and number of tests and control transfers were not mentioned. Following upon this discovery, delayed skin sensitivity to streptococcal M substance and tuberculin were accomplished, again using osmotically lysed or frozen and thawed cells from sensitive donors. The extracts were reported to have the same efficiency in transferring the allergy as did the same volume of live cells. Furthermore, ribonuclease (RNase) and later trypsin (Lawrence, 1959) did not affect the transfer by the extracts. It should be noted that ribonuclease is mainly effective against high molecular ribonucleic acids and that trypsin, in general, digests only denatured protein, so that, as Lawrence remarks, one cannot validly conclude that the substance responsible for the transfer is neither nucleic acid nor protein. The duration of sensitivity, as mentioned previously in connections with live cell transfers, among the recipients seemed to fall into one of several patterns. In one pattern the transferred sensitivity arose as early as two to four days and either persisted for long periods or faded. Another pattern showed a sensitivity which arose slowly until, after the lapse of some weeks, it became and remained strong generally for many months up to two years. The latter pattern is most easily reconciled with the idea of an active sensitization as opposed to or superceding actual hypersensitivity resulting from the transfer of cells. In the paper cited, control extracts were made from the cells of normal donors; one out of five recipients exhibited sensitivity that lasted for more than five months.

In a collaborative effort, Lawrence and Pappenheimer, 1956, (cf. Pappenheimer, 1955) reported transfer of delayed hypersensitivity to diphtheria toxoid with cell extracts. In general, the reactions of the recipients were rather weak (1+) at twenty-four hours after transfer, and maximal sensitivity appeared about nine days after transfer. It is interesting to note that almost none of the recipients produced any detectable antibody, which Pappenheimer feels indicates that antigenic material is not transferred in the extracts. Unfortunately, not all of the desirable controls were reportedly done. From this work, the term "transfer factor" (Pappenheimer, 1958) was coined to designate the principle in the leucocytic extracts responsible for conferring sensitivity upon the recipients.

A further bit of information concerning "transfer factor" accrued when it was observed that, if cells from a tuberculin sensitive individual were incubated with PPD, transfer factor was released into the medium and the cells lost their ability to transfer the hypersensitivity (Lawrence and Pappenheimer, 1957). When the cells came from a donor sensitive both to tuberculin and diphtheria toxoid, incubation of the cells with PPD released all the "transfer factor" for tuberculin hypersensitivity but the cells were still capable of transferring the toxoid sensitivity, indicating that the cells were not non-specifically damaged by the incubation. It is biologically curious, however, that a thirty minute incubation of 0.5 ml of packed white cells with 25μg PPD would completely desensitize the cells and release all the transfer factor. Unfortunately again, no control incubation of normal cells with PPD was performed to determine whether the antigen added to the medium contributed to active sensitization of the recipients.

Delayed-type hypersensitivity to fungal antigens has been transferred by Lawrence's group using leucocytic extracts (Rapaport et al., 1960a). In these experiments, sensitivity to coccidioidin was selected because infection with the fungus is highly endemic for a region in California. Consequently, cells from sensitive donors from the San Joachin Valley were obtained, frozen and transported to New York where they were repeatedly frozen and thawed and injected into coccidiodin-negative recipients. The design of the experiment was such that the probability that the New York recipients had had previous contact with the fungus would be low and the possibility of choosing recipients who had some pre-existing sensitivity would be very much lessened. When tested one day after transfer, none of the recipients gave a positive reaction, but at seven days one was scored as 2+, two as 1+ and one remained negative. At sixteen days, all had 2+ reactions. Using the technique of local challenge, seven of sixteen were positive at the third day and thirteen of sixteen were positive nine days after transfer. In control experiments, repeated testing of fifteen non-sensitive volunteers converted only one to give a positive reaction (Rapaport et al.1960b). In the control transfer of cell extracts from two coccidiodin-negative California donors into New York negative recipients, five out of eight recipients became positive one week after transfer, indicating in all probability the occurrence of active sensitization; one out of nine recipients became positive similarly when injected with cellular extract from one New York negative donor. The issue brings

to mind the experience of Jensen et al. (1962) with living cell transfers in which sensitivities not seen in the donors appeared to have been "transferred". The authors of the coccidiodin paper state, "Because of this finding we have not fully demonstrated that 'transfer factor' can confer upon the recipient de novo sensitivity". In addition to the ambiguity in these findings, specificity was not examined in this work. More proof is perhaps needed that the antigens of Coccidiodes immitis do not cross-react with those of some of the more ubiquitous fungi.

In an attempt to prove that a non-preexisting sensitivity can be elicited de novo in a normal recipient by means of cell extracts, Lawrence et al. (1962) established a succession of two to four skin homograft rejections in an individual, from whose cells an extract was prepared. The extract, considered to have 'transfer factor', was infiltrated around a graft of the same skin carried for three days by another individual. Accelerated rejection did, in fact, occur in the ensuing four to six days. Curiously, donor cells were not found to be active unless taken within a sharply defined period of time after the last sensitizing graft was rejected. The rejection was thought to be specific because skin from a "neutral" donor was not rejected before the tenth to twelfth days, as opposed to the specific rejection of six to nine days. Apparently, there is a real problem in humans of non-specific accelerated rejection of skin graft without any transfer of cells or extracts. Of 105 normal individuals grafted with skin randomly, Rapaport et al. (1962b) found that there were 16 second set rejections and 18 white grafts. Homograft antigens exist in leucocytic extracts, and in experimental animals (Billingham et al., 1958) these have been shown capable of sensitizing recipients to accelerated graft rejection. In fact, by fractionating the human leucocytic extracts by this method, Rapaport et al. (1962a) reported that successful active sensitization of human recipients to accelerated rejection of skin grafts by prior injection of the extract. It thus is not entirely proven that a "transfer factor" has been demonstrated in the human homograft system.

The evidence of Lawrence and his collaborators that a soluble material existing in leucocytes is responsible for the delayed-type hypersensitivities is supported by studies of Baram and Mosko (1962) and Freedman, Fisher and Cooke (1957) so far as the tuberculin system is concerned. Maurer (1960) has reported transfer of delayed-type hypersensitivity to chemically modified

human serum by means of leucocyte extracts. The phenomenology of the skin reactions occuring in the recipients is unquestionably real. The interpretation and of these reactions is, however, not unambiguous and the points which have yet to be clarified are these; that (a) the reactions are surely specific; (b) it be decided whether a process of active sensitization is involved or the results are purely passive transfer; (c) the sensitivity of the recipients be shown to appear de novo, rather than being merely the flaring-up of a pre-existing sensitivity.

At the outset, the features of transfer with cell extracts appear to be peculiar, in the light of current thinking on the relation of mononuclear white cells in the response to skin testing. A small number of cells drawn from the peripheral circulation appear to yield sufficient 'factor' to allow, after transfer, the adoptive sensitization of sufficient host cells to cause delayed-type reactions although few of the total mononuclear cells of the host go to a test area. The observed long duration of the hypersensitivity, to be explained in terms of a non-antigenic 'factor' would require that the 'transfer factor' remain for some months to years unexhausted by early tests.

We have pointed above to the hazard of re-exciting previously acquired but undetected hypersensitivities, either by serial tests or perhaps by special materials contained within cells or cellular extracts. Another difficulty is that the number of recipients of competent and non-competent cells, unlimited when working with experimental animals, is sharply circumscribed in human work; there is need to work with small groups of human recipients because of the danger of transfer of serum hepatitis with human leucocytes. Ideally, one would like to know the percentages of positive reactions in a statistical group of transfers using cells from non-sensitive donors into normal recipients, tested in parallel with the experimental individuals. This would be especially useful in evaluating the duration of sensitivity in recipients of cells or cell extracts.

2. Experiments with Non-Living Cellular Material in Animals

In his papers dealing with the cellular transfer of contact and tuberculin hypersensitivities, Chase (1945, Landsteiner and Chase, 1942) attempted to transfer with cells heated 30 minutes at 48°C or with frozen and thawed cells. Both treatments were found to abolish the ability of the cells to effect transfer.

The first recent suggestion that transfer of delayed-type hypersensitivity might be accomplished with non-living cells was made by Crepea and Cooke (1948). These workers claimed that, in two of three instances, dried spleen cells from guinea pigs sensitized to poison ivy extract were able to transfer the allergy. The authors even claim to have transferred the contact sensitivity in their guinea pigs with serum in seven out of 19 attempts, something that many workers have tried to do but have not succeeded. For this work which has not been confirmed, there exist several possible explanations besides true passive transfer. All the recipient animals were pretested with poison ivy extract one day before transfer, and tests were made one and two days after transfer so that the reactions observed occurred four days after the initial contact with poison ivy extract, a time when the first evidence of active sensitization would be expected. Secondly, the skin sites were depilated before testing, which treatment as will be shown in this thesis can often lead to non-specific reactions.

In 1954, Jeter, Tremaine and Seebohm reported the passive transfer of contact hypersensitivity to dinitrochlorobenzene in guinea pigs, injecting living peritoneal exudate cells and sonically disrupted cells intraperitoneally in parallel. Their results indicate that the disrupted cells transferred as well as, and in two instances, better than the whole cells, which gave only modest sensitivity. The activity was felt to reside in a supernatant fraction obtained by removal of sediment by low speed centrifugation. Large amounts of cells were required (donor:recipient ratios of 4:1 to 8:1), and positive results were variable, not being obtained in every transfer. They made similar attempts to transfer tuberculin hypersensitivity but they were not successful. It must be noted that all contact sites on the recipients were chemically depilated before the application of the test, and no tests were employed to ascertain whether the observed contact reactions were specific. In a later paper, this group (Jeter et al. 1957) found that electrophoresis of the cell extracts from guinea pigs sensitive to dinitrochlorobenzene or tuberculin contained an alpha globulin not present in extracts of exudate cells from non-sensitive animals. However, attempts to transfer the allergy using this alpha-globulin fraction itself have been unsuccessful (Jeter, personal communication). Further details of this work may be found in the thesis of one of Jeter's students (Long, 1960)

Cummings, Patnode and Hudgins (1956) claimed to be able to transfer tuberculin hypersensitivity in guinea pigs using sonically disrupted exudate cells. The reactions reported varied from 2 x 2 mm to 6 x 8 mm, whereas in the guinea pig, reactions to O.T., of less than 9 mm require experienced evaluation and description. Consequently, this writer feels that this work may well be disregarded.

Most recently, Turk (1961) reported the transfer of contact hypersensitivity to picryl chloride in guinea pigs by means of cell fractions of spleen and lymph node homogenates, sonically disrupted cells or even frozen and thawed cells. After centrifugation of the homogenates made in sucrose-Hanks at high speeds, the activity was found in the microsomal and "cell sap" fractions. Strong transfers (++++) were found with extracts from very few cells (5×10^8), and it appeared that the cell extracts or cell fractions were as effective as the living cells. Interestingly, no dose-response effect was found when increasing amounts of cell extracts were injected.

The results of work in this laboratory (Bloom, Chase and Goldstein, 1961) have indicated that the claims of Jeter et al. and of Turk could not be confirmed. In addition, Metaxas and Metaxas-Buehler (1955) and Bauer and Stone (1961) were unable to transfer tuberculin hypersensitivity with heated or frozen and thawed cells. Numerous other workers in this field have communicated to us that they had been unsuccessful in confirming the work of Jeter et al. and of Turk. In an extensive study of hypersensitivity reactions associated with homograft rejection, Brent et al. (1962) found that sensitized cells killed by heat, freezing and thawing, or by drying do not transfer passive local homograft reactivity. They were also unable to extract a "transfer factor" from peripheral blood leucocytes in the manner reported in man by Lawrence (1959). Interestingly, they observed that "disrupted leucocytes from blood and especially blood platelet-rich fractions of isologous origin can give rise to violent non-specific delayed inflammatory reactions which resemble tuberculin reactions superficially but differ from the histologically".

Very recently there appeared an intriguing report of Mannick and Egdahl (1962), claiming that phenol-extracted RNA obtained from lymph node cells of rabbits which have rejected a skin graft can "transform" normal lymphoid cells in such a fashion that, when they are injected into the original skin donor, a delayed-type reaction is observed. This "transformation"

appears to be specific, i.e. the cells injected into "neutral" rabbits fail to elicit as strong reactions as they do in the specific donor. Neither the normal cells nor the RNA from the sensitive cells alone can produce this reaction in the donor skin and RNase abolishes the effect of the RNA in converting the normal cells into active ones.

D. On the Nature of the Complete Antigen in Contact Hypersensitivity

The early work of Landsteiner and Jacobs (1935, 1936) established that simple chemical sensitizers formed conjugated antigens in the skin of animals. They found that the best sensitizing reagents had labile groups and were effective in coupling to amino groups in proteins. It seemed reasonable to assume that the complete antigen responsible for inducing hypersensitivity was a conjugate of the allergen and some skin proteins. That coupling occurs in the skin was shown clearly by Eisen and Tabachnick, (1958) who isolated C^{14}-dinitrophenylated amino acids from the extracted skin proteins of painted guinea pigs. To test the hypothesis that an allergen-protein conjugate was in fact the complete antigen, it seemed reasonable to couple allergens to soluble proteins in vitro and attempt to sensitize with the conjugates. When this is done, delayed-type sensitivity to the hapten is, in general, not obtained, but rather only antibody to the determinant group is found(Landsteiner and Chase, 1941; Eisen, 1959; Benacerraf and Gell, 1959a,b).

Some in vitro-prepared conjugates are, however, able to sensitize sensitize guinea pigs when given with killed mycobacteria and paraffin oil. The first successful conjugate consisted of picrylated guinea pig erythrocyte stromata or "ghosts" (Landsteiner and Chase, 1941). This method is still employed routinely in this laboratory to produce high levels of contact sensitivity. Objection to the conclusion that the conjugate stromata was, in fact, serving as the complete allergen has been raised by Eisen (1959) on the grounds that the allergen may simply have dissolved in the lipids of the cell walls, leaking out slowly in the animals. Thus Eisen suggests that free allergen itself was present and was responsible for the sensitization. The present writer believes that this objection is implausable for two reasons; first, after coupling in vitro, an enormous excess of alkaline glycine is added for the purpose of reacting with the unconjugated or unhydrolyzed allergen; and secondly, preparations of stromata which have stood for up to 17 years in aqueous suspension are undiminished in their capacity to sensitize.

Surely, in this time complete leakage of free PCl and its hydrolysis would have occurred.

The claim has been made by Mayer (1957) that the complete antigen in contact hypersensitivity is a conjugate of the allergen with "procollagen" obtained from skin. This "procollagen" mixed with free picryl chloride, blended in a water-in-oil emulsion and injected intraperitoneally, conferred a moderate sensitivity on guinea pigs which was comparable to that achieved with picryl chloride-plus-mycobacteria in adjuvant. Unfortunately, no data were given showing whether the "procollagen" coupled in vitro was able to effect contact sensitization and the possibility is therefore not excluded that this material may have served merely as an adjuvant enhancing the weak reactivity of picryl conjugates of guinea pig materials forming in vivo. In addition, Eisen and Tabachnick (1958) reported that, after painting guinea pigs with C^{14}-dinitrochlorobenzene, less than two per cent was present as conjugate in the epidermis at 24 hours, and that only traces were present in the corium, hence very little coupling to collagen could have taken place.

Recently, successful sensitization to contact reactivity has been reported with in vitro prepared soluble conjugates emulsified in complete Freund adjuvants. Benacerraf and Gell (1959 b) found that when a variety of allergens were coupled to homologous guinea pig serum albumin, after injection in adjuvant, contact reactions were obtained to the allergen painted on the skin. The contact reactions were less marked however, than intradermal reactions to the conjugated protein. When heterologous proteins were used as carriers, very poor skin reactions resulted even when large quantities of conjugate were administered. Curiously, dinitrophenyl conjugates even of homologous albumin did not produce contact sensitivity to dinitrochlorobenzene and this was supposed by the authors to account for the fact that Eisen was unable to find contact sensitivity in animals injected with dinitrophenyl conjugates. On the other hand, it should be pointed out that Chase finds dinitrophenylated guinea pig erythrocyte stromata to be quite effective in producing sensitization of dinitrochlorobenzene.

Salvin and Smith (1961) were able to sensitize guinea pigs to contact hypersensitivity without the necessity of using tubercle bacilli in their emulsion. The conjugate was specially prepared soluble extract of guinea pig skin coupled to dinitrofluorobenzene. The skin extract was prepared by homogenizing skin in a Waring Blender and coupling the supernatant obtained after

centrifugation of 30,000rpm. This finding remains the closest step to the desired goal, but it should be mentioned that in addition to soluble proteins in this supernatant material, there would be some contamination with cell membrane and membranes of the endoplasmic reticulum. If sensitization could be achieved without the necessary inclusion of paraffin oil and emulsifier, the requirement for duplicating the actual event that occurs when simple intradermal injections are made in the skin would have been met.

It thus appears that allergen-conjugated heterologous proteins are rather ineffective in sensitizing guinea pigs to contact hypersensitivity even if mycobacteria and paraffin oil are supplied, but that conjugates to homologous erythrocyte stromata or serum albumin will effect contact hypersensitivity in mycobacterial adjuvant; perhaps homologous skin extract, which does not appear to require mycobacteria, may not require paraffin oil but this has not yet been shown. As the result of some experimental observations to be reported in this thesis, an hypothesis will be presented which suggests that the complete antigen in contact hypersensitivity is associated with cell membrane materials.

E. Experimental Goals

From the foregoing discussion, it was apparent that a number of areas in delayed-type hypersensitivity in experimental animals required further investigation. The work described herein was initiated to shed some light on the following problems:

1. Can delayed-type hypersensitivity to simple chemicals or tuberculin be transferred in guinea pigs by means of disrupted cells, cells fractions or cells extracts, as claimed? The importance of this question is fundamental to an understanding of the phenomenon. If it were possible to transfer hypersensitivity by means of disrupted cells, then a biochemical entity existing in the competent cells, presumptively termed "transfer factor", which would be responsible for converting normal recipients to give positive reactions, might be isolated and characterized. An answer to this question would also bear on the problem of whether "immunologic information" possessed by competent cells could be incorporated by host cells and perpetuated in new generations by them.

2. What is the duration of acquired sensitivity in recipients after transfer of competent cells? Since 'cloning' of competent cells cannot

persist in outbred stocks of animals, information bearing on this question would tell whether any "instructional" material was transferred from competent cells to host cells leading to a sensitization of the recipients. It is the finding of Lawrence (1949) that, in the human, tuberculin sensitivity of recipients of living cells persists for up to two years and possibly indefinitely, while Metaxas and Metaxas-Buehler (1955) found, in the guinea pig, that transfer of tuberculin sensitivity is very short-lived in recipients of competent cells and they feel that the transferred cells themselves must participate in the reaction.

3. What is the effect of metabolic inhibitors on the transfer of hypersensitivity by treated competent cells? Some attempts were made to determine what metabolic activities of the living competent cells might be vital for retaining their competence, since it was found, as will be seen below, that cells rendered non-viable were unable to effect transfer. The objective was to inhibit specific aspects of cellular metabolism, e.g. oxidative metabolism or RNA synthesis, without killing the cells.

4. What is the nature and specificity of contact reactions in the guinea pig, both in actively sensitized animals and in recipients of competent cells? Because recent statements have cast doubt on the relative importance of allergen-directed specificity and carrier-directed specificity, it was of interest to show that, under the conditions used, the specific allergen could be distinguished from other chemical allergens which couple to skin in like manner. From this work, it was hoped that in experimental animals other chemical allergens could be used as irritants to detect non-specific cutaneous irritability without the danger of being misled by immunologic cross-reactions.

5. What is the nature of the "complete antigen" to which the immunologic mechanism responds in contact hypersensitivity when simple chemical allergens are painted on the skin? At the present state of our ignorance of delayed-type hypersensitivity, systems comprising respectively contact reactivations, tuberculin hypersensitivity, delayed reactions to soluble proteins and homograft rejection must be considered separately and generalizations about the phenomena are difficult to make. If more characteristics held in common by some of these systems were known, the task of unifying these separate manifestations of delayed-type hypersensitivity would be made less formidable.

II

METHODS AND MATERIALS

II. Methods and Materials

The general procedures previously established in this laboratory were utilized. No single description having been published, the techniques will be described at some length.

A. Animals

Rockefeller Institute albino stock guinea pigs, pen inbred, were used in most of these experiments (Chase, 1961). These animals had been freed of the common Group C streptococcal infection of commercial guinea pigs. For cell donors, animals weighing 600-800 g were selected, while 450-550 g guinea pigs were chosen as recipients. To eliminate histoincompatibility of the Y chromosome, cell transfers were made using animals of the same sex as donors and recipients.

B. Allergens and Test Reagents

1. Picryl chloride (hereafter designated as PCl): Commercial material (Eastman-Kodak) was recrystallized three times from hot absolute alcohol-benzene (2:1) after treatment with activated charcoal. M.p. 82-83°C.

2. Dinitrochlorobenzene (DnCl): Eastman Kodak Company material was recrystallized two to three times from absolute alcohol after treatment with activated charcoal. M.p. 50°C.

3. Ortho-chlorobenzoyl chloride (oClBCl): The Eastman-Kodak preparation had been redistilled at 76-77°C. *in vacuo* (5-6 mm pressure) and stored in sealed all-glass ampules.

4. Phthalyl chloride (PhthCl): A preparation from Eastman-Kodak was freshly redistilled *in vacuo*.

5. Para-Nitroso-N, N-dimethylaniline (NDMA): Obtained from Kahlbaum Company, Germany, this material was recrystallized from benzene. M.p. 83-85°C.

6. Maleic Anhydride (Mal.Anh.): Mathieson, Coleman and Bell crystalline material was used as received. M.p. 53-55°C.

7. 2-Phenyl-4-ethoxymethylene-5-oxazolone (Ethoxaz): This compound was synthesized by the procedure of Barber and Slack (1949) and kindly amplified by Dr. V. duVigneaud. Hippuric acid (22.1g), ethyl orthoformate (20.4g) and acetic anhydride (50 ml) were heated under reflux for three hours at 70-90°C.

The dark red solution was poured onto 200 ml. ice-cold distilled water to yield a precipitate. It was dissolved in benzene and in turn washed with ice-water and saturated NaCl solution. The benzene solution was dried over Na_2SO_4 and reduced to an oil by flash evaporation. The oil was dissolved in chloroform, treated with activated charcoal, and crystallized by addition of Skelly B (light petrolic ether). Three to seven recrystallizations were required to remove all red color. Yield was 2.2g (9% of theoretical yield).

Analysis: Calc. C. 66.35; H. 5.07; N. 6.44 M.p. (reported) 95-96°C.
Obs. C. 66.47; H. 5.08; N. 6.55 M.p. (observed) 95.5-96.2°C.

This compound was shown to be highly antigenic in rabbits by Gell et al. in (1946) and was used as an allergen by Turk (personal communication).

8. Tuberculin used as Purified Protein Derivative (PPD): Merck, Sharpe and Dohme. For the high concentrations that were needed (25μg per 0.1 ml), a special lyophilized preparation, free of lactose was kindly made available for these experiments by Dr. M. W. Chase.

9. Picrylated Myelin (P-myelin): Myelin from calf brain was prepared by Dr. W. G. Norton and Dr. L. Autilio* by homogenization and density gradient centrifugation in sucrose and generously donated by them for these experiments.

> A suspension of 25mg of lyophilized myelin in 5.0 ml saline was adjusted to pH 10 with 2 drops of 1 N Na_2CO_3 and 1.5 mg PCl in 0.5 ml absolute alcohol was added dropwise with stirring. The reaction was allowed to proceed for 15 min. at room temperature. Alkaline glycine (100mg/10ml saline, pH 9.5) was added to conjugate the unreacted PCl. After 10 min. the reaction was made neutral and the solution centrifuged in a screw-capped lusteroid tube at 8000 x g for 10 min. The bright yellow sediment was resuspended and washed twice with 10% alcohol-saline and finally three to five times with saline. It was stored in the cold in saline containing merthiolate 1:10,000. For injection, it was emulsified with an equal volume of complete Freund's adjuvant (Difco).

9. Picrylated Guinea Pig Erythrocyte Stromata (P-GPSt): The method of preparation has been described (Chase, 1954). The product was kept in the cold room in saline suspension with 0.25% phenol.

10. Solvents for allergens for skin testing were either virgin Spanish olive oil or dibutyl phthalate (Amend Drug and Chemical Co.).

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C. Sensitization

The methods used for sensitizing and testing animals have been described in detail by Chase (1954), and will be summarized here.

a) The "Combination Method" refers to the sensitization of guinea pigs by the injection of a water-in-oil emulsion, prepared aseptically, containing allergen-coupled guinea pig erythrocyte stromata ("ghosts") plus heat killed M. tuberculosis, Jamaica 22 strain, followed by two later skin paintings of the allergen at approximately day 20 and day 28. Animals are tested with dilutions of the allergen at about day 33 and cells are taken at about day 42.

> In a typical preparation, 3.9 ml medium grade paraffin oil (U.S.P., sterilized), and 1.1 ml paraffin oil containing 10 mg/ml tubercle bacilli (gently ground dry in a mortar to disperse clumps before adding the paraffin oil dropwise) and 2.5 ml molten Aquaphor (an emulsifying agent prepared from hydrolyzed lanolin obtained from Duke Labs.) were added to a sterile dry chamber and blended with a paddle connected to a motor driven stirrer. The paddle was rotated rapidly and 7 ml saline containing 11 mg P-stromata, pH 7.0-7.3, was added dropwise. The aqueous phase was added as rapidly as emulsification took place (approx. 30 min). Dispersed in this way, the emulsion had 0.75 mg tubercle bacilli and 0.75 mg PCl-stromata per ml. Each pig to be sensitized was injected with a total of 1 ml of emulsion into five sites of 0.2 ml in the nuchal area. The later applications of the corresponding simple allergen to the skin were made with 3 drops of 1% PCl or 1% DnCl in dibutyl phthalate (to prevent possible sensitization to the components of olive oil).

It is to be noted that guinea pigs develop hyperplasia of the regional lymph nodes by this method of sensitization.

b) The "Intradermal Method" refers to the sensitization of animals by intradermal injection of 2.5μg of the allergen/0.1 ml of saline, freshly prepared and given either as five simultaneous injections, or by daily injections of 0.1 ml for 12 to 14 times, sensitivity reactions appearing first at the injection sites in 5 to 7 days. The animals are allowed to rest and are tested about three weeks later.

> A stock 2% solution of PCl in absolute alcohol is diluted with alcohol to 0.3%. Of this, 0.2 ml is added dropwise with mixing to 24 ml of physiological saline to give a concentration of allergen of 25μg/ml. As PCl or DnCl are subject to hydrolysis in aqueous solution, the injection solution was prepared immediately before use.

c) The "Percutaneous Method", adopted from Chase's procedure by Jeter et al. (1954), was used in repeating their experiments in the course of

our work. Guinea pigs were painted with 5-7 drops of 2% DnCl in absolute alcohol on the same clipped skin area of the neck daily for 6 days. The animals were tested 11 days later and used for transfer purposes on the twenty-third day after initial treatment.

D. Testing for Level of Sensitivity and for Specificity

For estimation of the degree of hypersensitivity of actively sensitized animals, dilutions of the allergen solution were painted on clipped skin sites, and the resulting reactions were read at 24 and 48 hours.

> Areas on the back of the guinea pigs approx. 30 mm in width were freed of hair by means of an electric clipper with a No. 0000 blade having the cutting blades closely approximated. The sites were kept isolated by means of strips of unclipped hair approx. 5mm in width. On the anterior half of each area, one drop (1/35-1/40 milliliter) of the test solution was allowed to fall from a capillary pipette and was gently spread over a circular area of about 25 mm in diameter by a burnished glass stirring rod, taking care not to pass the rod against the direction of hair growth. Flat boots of adhesive tape were applied to the toes of the hind feet so that the animals could not scratch the test sites. Careful and gentle reclipping was done at 24 hours following an initial reading and sites were washed gently with wetted cotton pledgets as needed, particularly before photography. (There is no 'stationary phase' in the hair growth of the guinea pig.)

In general, animals sensitized to respond to contact with PCl by the "Combination Method" were tested with 1/15%, 1/50%, 1/150% and sometimes 1/450% picryl chloride in olive oil. Recipients of transferred cells or extracts or animals sensitized by the "Intradermal Method" were tested with 1% PCl while those sensitized by the "Percutaneous Method" were tested with 1%, 1/3% and 1/15% of the allergen (dinitrochlorobenzene) dissolved in olive oil.

The reactions were examined, preferably by daylight, and the intensity of reactions was scored by the following system (Chase, 1954):[1]

Score	Appearance
++++	red, generally markedly thickened
+++	reddish-pink, sometimes macular, slightly thickened
++	pink or pale pink, confluent to edge of test area
+	faint pink, confluent to edge of test area
±	very faint pink, confluent; or, faintly pink but patchy or uneven
tr	a trace of erythema, not confluent

[1]The gradation in observed reactions is shown in Plate I.

For exquisitely hypersensitive animals, this scale has been expanded to include:

Score	Appearance
+++++	bright red, well thickened
+++++	very bright red, very markedly thickened with large patches ("Plaques") of islands of epidermis separated from the cutis (superficial necrosis).

Tuberculin sensitivity of recipients was determined by reading the diameter, color and quality of reaction appearing after the intradermal injection of 20-25μg PPD on the flank. (Actively sensitized animals in these experiments were not tested with tuberculin. Other animals sensitized similarly by the "Combination Method" in this laboratory and tested routinely exhibit uniform sensitization to 0.5μg test of PPD, the reactions measuring 13-17 mm, mostly with central discoloration and necrosis, and marked induration.)

In testing for specificity of reactions, a variety of allergens was used, the concentration of each chosen to be relatively high, approaching closely that concentration which was just slightly irritative to the skin ("limit of toxicity"). The most sensitive indicator appeared to be 15% o-chlorobenzoyl chloride in olive oil. Other test solutions employed were 1/5%-1/15% phenylethoxymethylene-oxazolone in dibutyl phthalate, 8%-15% maleic anhydride in dibutyl phthalate, 2%-4% phthalyl chloride in olive oil and 0.75 per cent p-nitrosodimethylaniline in olive oil.

E. Induction of Peritoneal Exudates

The principle method employed to produce mononuclear exudates (Chase, 1945) was the intraperitoneal injection of 25 to 28 ml sterile Bayol F paraffin oil (Humble Oil and Refining Co.). Cells were harvested 48 hours later.

On occasion, 1 ml of 12% sodium caseinate in saline was injected into the peritoneal cavity and a mononuclear response was obtained on the third elapsed day.

A third method occasionally used in animals sensitized by prior intraperitoneal injection of tubercle bacilli in paraffin oil consisted of injecting 0.2 mg heat killed M. tuberculosis (Strain Jamaica No. 22) suspended in saline, intraperitoneally, exudate cells being harvested on the following day.

F. Basic Media for Handling the Cells

The basic medium for obtaining and handling the cells was Hanks balanced salt solution (1949), prepared with phenol red and sterilized by Berkefeld filtration. Gelatin was added to 0.125% for the purpose of increasing the viscosity and colloid osmotic pressure of the Hanks medium for the protection of the cells (Rous and Turner, 1916).

> A special gelatine prepared from beef bone by the KOH method (Knox Gelatine Protein Products, Inc. Type 1592) was dissolved to 5 per cent, neutralized to pH7.2, autoclaved at 15 pounds for 10 minutes, and quickly chilled to preserve viscosity. This stock was melted and added to a concentration of 0.125%.

The Gelatin-Hanks medium (G-H) was employed for handling cells at all stages but it was modified for collecting cells in peritoneal exudates (II-G) by addition of heparin (Connaught Laboratories) 1:70,000 and, 4 ml 1.4% aqueous $NaHCO_3$ per 250 ml solution (autoclaved but not regassed with CO_2). Correction of pH, seldom required, was made by adding dropwise 1.4% $NaHCO_3$ solution that had been autoclaved and regassed to proper pH.

For the maintenance of cells for prolonged periods and for tissue culture, Trowell's Medium (Trowell, 1955) and Eagle's Medium (Eagle, 1959), the latter being obtained from Microbiological Associates, were used with inactivated guinea pig serum in the concentrations noted for the particular experiment.

G. Procedure for Obtaining Cells for Transfer

A typical experiment will be described for obtaining peritoneal exudate, lymph node and spleen cells. All of these cell sources were utilized in most, but not all, experiments. The procedures were conducted throughout with sterile technique, Four persons were needed for efficient collection. One person anesthetized and exsanguinated the donor guinea pigs; another performed the initial dissection of the skin and superficial muscle; a third harvested the peritoneal exudate cells, the fourth removed the cervical and axillary lymph nodes and spleen. The timing was such that the next animal was ready as one operation was completed on the previous one.

The donor animals were first prepared by clipping the hair from the ventral surface with an electric clipper. The animals in turn were anesthetized with chloroform, the major vessels and muscles at the base of the skull were

severed, the cervical vertebrae were cut with a bone cutter and the pigs were bled from the neck suspended by the hind legs. The second worker pinned the animal to a board, ventral surface upward, sponged the clipped skin with alcohol and aseptically reflected the skin and superficial fascia from the pubic symphysis to the jaw, leaving a sterile clean surface. The animal was covered with waterproof cellophane to avoid drying of the tissues.

The exudate cells were obtained by the third worker as follows: A midline incision of about 2" was made close to the umbilicus, the peritoneal wall and the opening was retracted and lifted by Pratt T forceps hooked over split-rings attached to ring stands, thus making a well of the peritoneal cavity. The peritoneal cavity was washed in turn with 50ml of washing fluid and then 30 ml. The washing fluid consisted of gelatin-Hanks solution modified as described above by adding 4 ml of 1.4% autoclaved but unregassed $NaHCO_3$ per 250 ml fluid medium and heparin to 1:70,000. The extra portion of carbonate was needed to correct the acidity of the peritoneal exudate which exceeded the buffering capacity of Hanks solution. After the fluid was introduced into the peritoneal cavity, the intestines were gently agitated to free cells, and the fluid was removed with a 10ml pipette inserted into a stainless steel tube having perforated sides and tip to prevent clogging of the pipette by the greater omentum. The cells harvested from each animal were transferred to a sterile separatory funnel closed by a neoprene tubing and clamp, in order to separate the paraffin oil from the aqueous layer containing most of the cells. After the exudates had been harvested, the cell suspension was withdrawn into 50ml screw-capped tubes and the cells were sedimented at 1100-1300 rpm (230 x g) for five minutes in an International Centrifuge, Size 2, at room temperature. The paraffin oil was carefully aspirated from the top of the tubes by a sterile 2ml pipette connected to a water pump, and then the medium was stripped off the cell pellet. The packed cells were resuspended in standard gelatin-Hanks solution by gentle pipetting with a 2ml pipette, pooled and distributed in clean tubes to contain approximately 0.5ml packed cells per tube, and were centrifuged at 900-1100 rpm for five minutes. In general, the cells were resuspended in 1-2ml gelatin-Hanks per 0.1 ml packed cells and passed through two nested strainers - 100 mesh stainless steel (fine) and a nickel "Lectromesh" screen (0.003" hole size) (very fine) to remove clumps. (Figure 1) An aliquot of cells was removed for white cell count. The cells were then ready for injection or further manipulations.

After the peritoneal exudate cells had been harvested, the fourth worker removed three pairs of cervical, one pair of sub-maxillary, the axillary lymph nodes and sometimes the mesenteric lymph nodes as well. Two pair nodes were removed with their fat pads and kept moistened with gelatin-Hanks solution until the cells were worked up. The nodes were carefully trimmed of fat and the cells were allowed to escape by gentle teasing of the nodes under gelatin-Hanks fluid with two- and three-tined dissecting needles (Figure 1). The nodes were not minced. The cell suspension was passed through the fine and very fine strainers described above, and then washed in the manner described above for the peritoneal exudate cells. The lymph node cells were worked up as soon as they had been freed from fat since they appear to be labile and lose effectiveness in transfer capacity with careless manipulation and with standing.

The spleens were removed last and until worked up were stored in the refrigerator under a layer of sterile paraffin oil. The spleens were trimmed from mesentery, freed of paraffin oil by flotation of the oil, washed well with gelatin-Hanks, blotted, weighed and remoistened. Splenic cells have been obtained in two ways. One method was to mince the spleens finely with a scissors in small amounts of gelatin-Hanks. Recently, greater yields have been obtained by the technique described by Turk (1961) and demonstrated by him to us. Narrow strips sliced from three to four spleens were placed on a square of sterile nylon stocking, double thickness, that had been wetted with gelatin-Hanks solution. The cells were obtained by gently pressing the spleen slices in the stocking into gelatin-Hanks, the manipulator wearing sterile rubber gloves. The cells were strained and washed as above. Sometimes, it was desired to reduce the large numbers of contaminating red cells and a modification of Turk's procedure (1961) was found to be effective. The washed and packed pellet of splenic cells was suspended in 20 ml of 0.05M NaCl (one-third isotonic for just 75 seconds. Isotonicity was restored by the addition of 1.25ml 10% NaCl with swirling. Twenty milliliters of gelatin-Hanks was then added and the cells were centrifuged at 230 x g for five minutes. The cell pellet was usually light brown or white, and the erythrocyte stromata remained in the supernatant. Splenic cells were washed two or three times before use.

For an experiment employing fifteen to twenty donor animals, the time from sacrificing the first animal until exudate cells were injected or used

Figure 1

SPECIAL TOOLS USED IN OBTAINING SINGLE CELL SUSPENSIONS

for further manipulation was approximately 3-3.5 hours. The corresponding lymph node cells required approximately three hours for trimming from fat, teasing the cells and washing. Splenic cells were regularly processed in two hours. It was usually possible, with four workers, to conduct some of these operations simultaneously.

Average yields from the various cell sources are given in Table I.

Table 1

YIELDS OF WHITE CELLS FROM SENSITIZED GUINEA PIGS

Cell Source	Cell Volume/donor	Cell Count/donor
A. Sensitized by the Percutaneous Method†		
peritoneal exudate*	0.12	3.4×10^8
lymph node	0.05	2.0×10^8
spleen	0.11	4.4×10^8
B. Sensitized by the Combination Method		
peritoneal exudate*	0.12-0.14	$1.5\text{-}2.5 \times 10^8$
lymph node	0.11-0.13	$4.0\text{-}6.0 \times 10^8$
spleen cells	0.20-0.30	$3.5\text{-}5.0 \times 10^8$
	0.14-0.16(rbc laked)**	

† - Average yields from only two experiments

* - Paraffin oil (Bayol F) induced 48 hour exudate

** - Laked by the method given in Section II-G

H. Sonic Disruption of Cells

Three different instruments were used for the sonic disruption of cells, namely, a Raytheon 10kc, 200W model[1] (an older model kept in excellent condition), a Raytheon 9kc, 50W model,[2] and a British "MSE" 18-22kc, 60W instrument[3] (Medical and Scientific Engineering). To prevent bacterial contamination of the cellular material, the chambers, gaskets and caps of the Raytheon oscillators and the probe and gasket of the MSE instrument were soaked in 70% alcohol and rinsed thoroughly with sterile saline.

1 Courtesy of Dr. W. F. Goebel
2 Courtesy of Dr. I. Abrahams, Department of Microbiology, Cornell Med. College
3 Courtesy of Dr. K. Lawrence

The oscillator most frequently used was the 10kc, 200W Raytheon, and the cell suspensions (not over 7.5ml) were contained in single tubes (sterile "Hi-Temp Polyethylene", capped, 17 x 100 mm) held at an angle within the fully closed sonication chamber containing crushed ice and water above the level occupied by the cell suspension. This method, which will be referred to on occasion as "indirect sonication", allowed excellent control of the process of rupture. Disruption of all cells occurred usually in 5 to 7 minutes, but variation was made in the time of exposure of cells to sonic vibrations between 15 seconds and 15 minutes. The effective times were shorter when the other oscillators were used in which the cells were in direct contact with the transducer (referred to as "direct sonication"), but all apparatus could be used to secure equivalent cellular damage.

In general, cellular disintegration was carried out at a starting temperature of 0°C. with ice-snow present. A temperature rise of only 4 to 6° occurred with the 10kc, 200W Raytheon model, and the temperature was reduced to 0° before and in the course of each run. In a few cases, temperatures of 17°, 30° or 40° were used.

Cell disruption was followed in all experiments by phase contrast microscopy at intervals, and estimations of cell viability were obtained by staining one or two drops of the chamber contents diluted in four drops serum with one drop 2% trypan blue.

I. Disruption of Cells by Freezing and Thawing

Cells were frozen and thawed in the presence of desoxyribonuclease by the general method of Lawrence (1955). Cells were suspended usually to 5% concentration (v/v) in Hanks or gelatin-Hanks solution containing 0.1 or 0.25mg crystalline DNase-I per milliter (Worthington Biochemicals). The cells were placed in stainless steel tubes, capped with expanding neoprene gaskets, and lowered for 10-15 minutes in a dry ice-ethanol bath at -68°C and were then thawed by holding briefly in a 37°C. water bath until just melted. The process was repeated for no fewer than 7 cycles. Again, the extent of disruption was followed by phase contrast microscopy.

J. Homogenization of Cells and Preparation of Cell Fractions

Free cells or tissue slices were homogenized in a sterile Potter-Elvehjem tissue grinder (150mm tube) kept in an ice-bath, with a teflon pestle connected

to an electric motor. The tissue was homogenized for ten minutes, and if necessary, the residual tissue after centrifugation at 230xg for five minutes was rehomogenized in fresh medium.

Homogenates made in hypertonic sucrose medium were then centrifuged at 5,000 x g. (7,500rpm) in the cold to remove unbroken cells and cellular debris. The supernatants were centrifuged at 100,000 x g (39,000rpm) for 60 minutes in the Spinco Model L ultracentrifuge at 4°C. The final supernatant fraction will be referred to as the "Cell Sap", and the pellet as the "Microsomes". All operations were carried out aseptically, the lusteroid tubes for the high speed centrifugations being sterilized by soaking in "Zephiran" chloride, 1:1000.

K. Route of Transfer of Cells or Cell Extracts

Cells or cell extracts were injected into recipients either intravenously via the jugular vein or intraperitoneally. Volumes not greater than 5.0 ml were injected intravenously to avoid overly distending the vessels, but on occasion up to 10 ml cell sap have been injected slowly. It should be pointed out that the cells were always strained again through the finest strainer immediately before intravenous injection to prevent incipient clumps that could cause death of the recipient from thrombi. In several experiments, cell extracts were injected intradermally in 15-20 sites (0.2ml/site) on one side of the animal's back, leaving the other side free for the making of contact and intradermal tests.

III

ATTEMPTS TO TRANSFER DELAYED-TYPE HYPERSENSITIVITY BY NON-LIVING CELLULAR MATERIAL

III. Attempts to Transfer Delayed-Type Hypersensitivity by Non-Living Cellular Materials

The system of readings and the arbitrary scale employed for recording the level of reactivity of animals to contact test in these experiments has been presented in II-D. If there is any reactivity at all, what must be decided is whether the reaction present in the skin is attributable to a specific hypersensitivity or to non-specific irritability, and in either case, a judgment as to the extent or degree is sought. No particular problem is raised in testing and evaluating specific reactions on an actively sensitized animals under normal conditions of testing. Indeed, non-specific allergens or irritants such as cantharidin will not mimic such reactions, which arise in a characteristic time sequence and show gradually ascending induration and infiltration, although some degree of marginal edema may be present. When, however, one tests with dilutions of allergen which might be expected to produce weak border-line reactions, e.g., 1/150% PCl on animals sensitized by the "Combination Method", the evaluation is contingent upon several factors. Several types of artifacts can arise in contact testing: irritation of the skin arising from clipping, possible threshold irritation already existing in the dermis, improper testing technique in making contact tests (stroking the hair stubble against the direction of its growth), toxicity of the allergen in the concentration used, and toxicity of the vehicle in which the allergen is dissolved. Because of these possible sources of error, it is good routine practice to test normal animals as "toxicity controls" in identical manner to the experimentals. When low degrees of sensitivity are to be evaluated, it is always necessary to include toxicity controls. When all factors are taken in account, the fact remains that a few normal guinea pigs are subject to untoward responsiveness to some allergens used close to their toxic threshold concentrations. A very few individuals of our stock perhaps 1%, ever show a reaction of the "one-plus" (+) degree to 1% picryl chloride or 1% dinitrochlorobenzene in olive oil, yet about 20% show some very faint degree of erythema. Undoubtedly there are differences in skin quality among guinea pigs, probably inherited, which are involved in these slight toxic reactions. Therefore several guinea pigs should be included as toxicity controls.

It becomes still more difficult to make proper judgment about the hypersensitivity of individual animals that have received cellular materials

and give reactions upon testing as high as ' ± ' or slightly greater degree. The possibility that they are no longer physiologically normal animals may change normal relationships. For example, rabbits have been observed to release C_x-reactive protein into the blood stream in sharp spikes within a few hours following transfer of both living and killed rabbit white cells (Chase, M.W., personal communication).

Another factor which must be taken into account before minor reactions can be evaluated is that of specificity. In some of the work to be described, only low-grade responses were encountered, and it soon became apparent that it was essential to be able to differentiate between specific hypersensitivity and non-specific reactivity in particular recipients of cellular and subcellular materials. Recourse was had to a technique that Chase (personal communication) had used previously in a rather special case[1]. Skin testing was conducted not only with the specific allergen in question, but also in parallel with a chemically distinct allergen, at a concentration approaching its toxic limit, e.g.,15% o-chlorobenzoyl chloride in olive oil. Justification for the choice of this reagent and for our interpretation of reactions to this compound as non-specific reactivity is presented in Section IV-C. We have found that the results of simultaneous testing with oClBCl and the specific allergen on recipients of cellular materials has often been helpful in determining whether a non-specific component of reactivity was present. Despite the undoubted value of this procedure, it must be pointed out, however, that the capacity to respond to 15% oClBCl as an irritant varies from one animal to another and that the problem of non-specific reactivity is not solved in all instances.

The criteria which were brought to the evaluation of skin reactions in terms of hypersensitivity are, thus, that the reactions be above the general leve of toxicity and, in particular, well above the reaction of normal toxicity controls, and that they be specific for the sensitizing allergen (III-F).

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[1] This technique was introduced in studies on a unique disease of recipients of living white cells termed "lymph node disease" or "erythematous disease". This condition sometimes occurs after cell transfer owing to histo-incompatability between donor cells and recipient (Chase and Battisto, 1955; Battisto, in detail, 1961).

The procedure which will be employed in presenting the results is as follows: All trace reactions (scored as ' tr ' or less), when individual normal animals tested as toxicity controls gave similar reactions, are considered negative. Reactions scored ± or greater are considered as not negative (questionable) and are evaluated case by case with respect to hypersensitivity according to the above criteria.

A. Transfer of Intact but Non-Viable Cells

It was shown by Chase (1945) that competent cells lost their capacity to transfer tuberculin hypersensitivity or contact sensitivity towards chemical allergens by heating at 48°C for 15 minutes, or by freezing and thawing. In an unpublished observation, Chase found that the ability to transfer was also destroyed by incubating competent cells for two hours or less in chloroform-saturated Hanks solution. The latter observation was confirmed in the course of these experiments: When living cells from donors sensitized by the "Combination Method" were incubated for 30 minutes in chloroform-saturated gelatin-Hanks or gelatin-saline-citrate and then resuspended in fresh medium, it was found that they were no longer able to transfer hypersensitivity to picryl chloride or tuberculin when injected intraperitoneally into recipients. It may be added that no loss can be said to occur through the discarding of the killing menstruum, since Chase (personal communication) injected his cells in the killing menstruum itself and this was done in some of the present experiments.

In addition, incubation of competent cells with 10^{-3}M mercuric chloride in saline for two hours at room temperature and resuspending them in fresh saline for injection abolished their ability to transfer contact or tuberculin hypersensitivity.

In two experiments, competent cells were lyophilized, and when resuspended and injected into normal recipients, it was found that their capacity to transfer hypersensitivity had been destroyed.[1]

After all of these treatments, it was found that the nuclei of the cells stained with trypan blue indicating that the cells had been rendered inviable.

In summary, one abolishes the ability of immunologically, competent cells to transfer delayed-type hypersensitivity by heating the cells at 48°C, by

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[1] DnCl 284-A, R 14; PCl 473-A, R 17 (Appendix Tables II-b, VII-a).

freezing them, as previously reported, and also by treatment with chloroform-saturated buffer, mercuric chloride, or by lyophilization.

B. Transfer of Disrupted Cells and Subcellular Material

1. General Considerations

In this section will be presented attempts made in this laboratory to transfer delayed-type hypersensitivity by means of disrupted cells or cell extracts. The starting point for the resumption of such studies was a series of experiments designed to repeat the published reports of Jeter et al. (1954) that transfer of contact hypersensitivity could be accomplished with sonically disrupted peritoneal exudate cells. A great variety of experimental variations has been employed, in the use of three methods for sensitization of the donor guinea pigs, the use of 13 different media and three different methods of disrupting the cells, for example. The work was initiated by Drs. Gerald Goldstein and M. W. Chase, and the present writer collaborated in the first five experiments[1] and then undertook its extension.

In addition to the criteria put forward early in this section for what, in our judgement, constitutes a positive contact reaction, we cite here three principles which were applied to the special case of transfer with cell extracts: a) transfers of subcellular material to be judged positive should render recipients definitely sensitive in proportion to the amount of material transferred, i.e., the response should be dose-dependent; b) chemical depilation should not be necessary to observe positive reactions, since it is not required when living cells are transferred; and c) one might expect the efficiency of disrupted cells to be lower than that of living cells.

It has proved convenient to express the amount of cells or subcellular material in terms of a donor:recipient ratio (D/R), i.e., the number of donors which contributed cells of a given source to one recipient. For example, exudat cells used in a 4:1 ratio indicates that four guinea pigs contributed the peritoneal exudates that were ultimately transferred into one recipient. Obviously, when large pools of cells are prepared, the ratio indicates the equivalent number of donors as the proportional part of the pool used. The donor:recipient ratio also gives an indication of the amount of cells transferred since the

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[1] DnCl 280; PCl 462, 463, 464, and 465.

yields of cells per donor, in terms of packed cell volumes or counted cells, from animals sensitized by the "Combination Method" are sufficiently constant (See Table I for average yields from the various cell sources). Packed cell volumes were measured for each experiment, and for most transfers cell counts were performed as well. Data on the yields of cells from each source in the various experiments and also the method of sensitization, time of sacrifice, and time of last test of the donors are presented in Table 4.

It may be helpful to bear in mind that the minimal number of living cells which consistently gave strong positive transfer reactions was on the order of donor:recipient ratios of 0.5:1 to 1:1 (or approximately 1 to 1.5 x 10^8 peritoneal exudate cells, 3 to 5 x 10^8 spleen white cells, and 4 to 6 x 10^8 lymph node cells). Many times this number was generally transferred in the form of disrupted cells or subcellular material.

Cell transfers from sensitized donors to normal recipients were made in about thirty-three large experiments under varying experimental designs and with donors sensitized in one of three ways. Pools of cells from each individual cell source obtained from 15-25 donors were divided and handled in different ways. In the experiments in which transfer of disrupted cells were investigated, 128 recipients were used and 64 normal animals served as toxicity controls.

In every experiment but three, the ability of the living cell to effect transfer was actually demonstrated with one or more of the cell pools, and in the remainder the methods of sensitizing the donors and obtaining cells were no different so that the cells were expected to be fully competent to transfer. There was no case in which a transfer was attempted with disrupted cells that, given living, would have failed to transfer. The various uses to which individual cell pools were put, therefore, represent the equivalent of several separate transfer experiments, each valid in its own right.

Table 2 shows the number of portions of living cells from each cell source transferred as extracts, each portion being competent to transfer as living cells. The number of recipients into which the disrupted material was transferred or which received centrifugal fractions of the extracts is also given.

The method of presenting experimental data is as follows. Transfer experiments are listed in Table 4, with the method of sensitization of the donor

Table 2

CELL POOLS UTILIZED FOR PREPARATION OF TRANSFERRED SUBCELLUAR MATERIAL

Experiment	Cell Sources					
	Peritoneal Exud. Cells		Lymph Node Cells		Splenic Cells	
	Portions handled	No. of Recipients	Portions handled	No. of Recipients	Portions handled	No. of Recipients
DNC1 280	3	3	-	-	3	3
283-A	3	5	2	4	-	-
283-B	1	2	1	1	1	1
284-A	2	4	1	2	1	1
PC1 462	2	2	-	-	2	2
463	2	3	2	2	2	2
464	4	4	3	5	2	2
465	2	2	1	1	2	2
466	2	2	1	1	1	1
467-A	3	4	3	3	1	1
472	-	-	1	1	4	4
473-A	4	6	-	-	-	-
477	2	3	4	4	2	3
487-A	3	4	5	5	4	4
491-A	-	-	3	3**	3†	3**
492-A	-	-	4	4**	4†	3**
492-B	-	-	4	4**	4†	4**
516/Tb 137	1	1	1	0.5**	1	0.5**
Totals	34	45	36	40.5	37	37.5

The 108 portions used were multiples of the amounts that, as living cells, were effective in transferring strong contact-type reactivity. The number of recipients shown is 121. Transfers of living cells to verify the competence of the cells were performed in addition to the above, on at least one cell source in each experiment except for PC1 463-5.

† Splenic cells freed of red blood cells by suspending in 0.05 M isotonic saline for one minute only, then restoring isotonicity and centrifuging.

** Lymph node slices and spleen wbc were pooled. Half of the recipients used are assigned arbitrarily to each cell source.

TABLE 4

ORIGIN OF CELLS USED IN TRANSFER EXPERIMENTS, EXPRESSED AS YIELDS PER DONOR

Expt. No.	Method Sensitized	Day Used	Days After Test	Number Animals	Peritoneal Exudate ml. Cells	Peritoneal Exudate Cells n x 10^8	Lymph Nodes ml. Cells	Lymph Nodes Cells n x 10^8	Spleens ml. Cells	Spleens Cells n x 10^8
PART A. SENSITIZATION TO PICRYL CHLORIDE										
466	Comb.	43	3	15	0.075	----	0.12	----	0.17	----
467-A	Comb.	48	9	22	0.16	----	0.16	----	0.24	----
472	Comb.	44	7	16	0.19	----	0.18	----	0.22*	----
473-A	Comb.	44	9	17	0.14	----	0.14	----	0.15	----
480-A	Comb.	44	7	5	0.16	----	ND	----	0.16	----
480-B	"	52	15	5	----	3	ND	ND	ND	ND
477	Comb.	42	6	20	0.14	4.1	0.11	----	0.11	3.0
483-A	Comb.	54	9	5	0.14	2.8	ND	ND	ND	ND
487-A	Comb.	37	6	16	0.10	1.7	0.11	6.5	0.17	3.7
487-B	"	44	13	14	ND	ND	0.15	5.1	0.24	4.7
491-A	Comb.	39	5	11	ND	ND	----	3.6	----	1.3
491-B	"	62	28	9	0.12	2.7	0.12	5.6	0.20	3.5
492-A	Comb.-Sp.††	33	6	11	ND	ND	----	3.6	----	3.0
492-B	" "	35	8	10	ND	ND	(wt.Ⱡ.36g)		----	----
497-A	Comb.**	54	21	8	----	1.0	----	----	----	3.3
497-B	"	61	28	10	----	0.80	----	----	----	----
500-A	Comb.	63	18	14	0.1	2.5	----	----	0.31	5.0
500-B	"	64	19	5	0.11	2.32	----	----	0.31	6.4
503-A	Comb.	62	27	8	0.17	2.6	----	----	0.31	4.5
503-B	"	68	9	10	0.12	----	0.11	----	0.55	----
507	Comb.	69	19	12	0.09	2.4	0.11	5.5	0.27	5.4
516	Comb.	24	not tested	12	----	1.45	(wt.0.71g)	2.1	0.3	----
518	Comb.	64	24	8	0.15	2.0	ND	ND	0.29	5.5
519	Comb.	45	12	21	0.14	2.4	0.24	8.4	0.38	6.6
523-A	Comb.	57	14	20	0.10	1.8	ND	4.5	----	----
523-B	"	62	19	10	0.13	1.6	(L.N. & Spl. Cells, total 8.8 x 10^8)			
PART B. SENSITIZATION TO DINITROCHLOROBENZENE										
280	Percut	22	5	30	0.15	----	ND	ND	0.09	----
283-A	Percut	23	6	23	0.09	3.4	0.05	2	0.14	5.4
283-B	Comb.	50	14	10	0.13	2	0.15	6.5	0.12	3.3
284-A	Comb. ***	44	6	20	0.15	2.6	0.14	4.2	0.16	4.8
284-B	Tbc+GP Stroma	42	--	12	0.13	---	ND	ND	0.16	----
285	Comb.	46	9	6	0.12	1.1	ND	ND	ND	ND
287-B	Percut	162	4	8	ND	ND	ND	ND	0.10	1.25

Legend: Animals were Rockefeller Institute albino pen-inbred stock excepting that Hartley strain guinea pigs were used in PCI-497. Males were used both as donors and recipients excepting in PCl 503. Unless otherwise indicated, sensitization was effected by the "Combination Method." Percut, sensitization effected by 5 applications, one each day, of dinitrochlorobenzene in alcohol, made at base of neck. ND, not done.

†† - Sensitization effected by the "Combination Method", but in accelerated fashion.

** - Hartley strain guinea pigs used.

*** - Laked and processed guinea pig erythrocytes (GPSt) but not coupled with chemical allergen were suspended in saline and emulsified with killed M. tuberculosis, paraffin oil, and Aquaphor.

Detailed protocols for each experiment in which disrupted cells were transferred appear in Appendix Tables I through XI. Summaries of the experiments are given in Table 6 for the transfers of sonically disrupted cells and in Table 7 for cells disrupted by freezing and thawing or homogenization. In the original protocols will be found the sources used, the methods of handling the cells, routes of transfer, times of testing and reactions noted after each test. Also present, there, is the data for certain recipients which received living cells to validate the competence of the transferred cells and for the normal animals included for toxicity controls in each experiment.

2. Transfer of Sonically Disrupted Cells

The experimental variables that were applied to sonically disrupted cells will be discussed separately, although sometimes two or more parameters were varied in a single experiment. All of these reflect our concern to explore fully the possibility of securing effect in subcellular transfer material.

a. Media Employed

Various media were employed with the hope of protecting constituents that might otherwise be labile. These are listed below and are summarized in Table 6, which shows cell ratios used in each transfer and the type of sonic disruption.

i). Gelatin-Hanks (G-H): This was the basic medium used for obtaining and handling cells (II-F), and was used in 41 transfers.

ii). Serum-Hanks (S-H): To include the possibility that the presence of a serum component might be necessary for the preservation of a liberated factor or to potentiate its utilization by the recipient, Hanks solution containing 7-15% homologous serum, sometimes freshly drawn or often containing at least one-tenth part fresh serum to provide a source of complement, was employed with additives to be described, in 17 transfers.

iii). Serum-Hanks with Ribonucleic Acid (S-H + RNA): In the event that a ribonucleic acid was involved in the transfer of hypersensitivity and subject to destruction by liberation of intracellular ribonucleases upon sonic disruption of the cells, commercial yeast ribonucleic acid to a concentration of 1% (Schwartz Biochemicals) was included in the disruption medium to serve as a competitive substrate for ribonucleases.

iv). Serum-Hanks with Ribonucleic Acid and Cysteine (S-H+RNA+Cys): Cysteine (0.01M) was included in 18 transfers to protect components of the extract which might be labile to oxidation.

v). Hanks with sucrose (H+Sucrose): Hanks solution was made hypertonic with sucrose to a concentration of 0.35M in one experiment.

vi). Hanks solution lacking magnesium and calcium ions: Hanks solution was prepared without magnesium and calcium ions for one experiment to eliminate possible inactivation of a factor by the binding of these divalent cations.

vii). Gelatin-Saline-Citrate (G-S-C): As described in II-F, citrate was added in an attempt to chelate divalent ions.

viii). Gelatin-Hanks containing tuberculin (G-H + PPD): Spleen cells were sonicated in one experiment in the presence of 50μg PPD to ascertain whether the antigen might protect a factor responsible for the transfer of tuberculin hypersensitivity.

b. Times of Exposure of Cells to Sonic Vibrations:

During sonication, all cell extracts were examined under phase contrast microscopy at intervals and the trypan blue method (II-H) was used to detect cells with stainable nuclei (non-viable cells). The oscillators used are described above (II-H). The effectiveness of disruption varied with time of treatment, the density of the cell suspension and the cell type used. Lymphocytes, for example, proved to be rather resistant, while large mononuclear cells were quite susceptible to disruption. Sonication was continued until the cellular material was reduced to the point desired. It is to be mentioned that Jeter et al. (1954) and Long (1960), using direct sonication in the chamber of a 9kc, 50W Raytheon, sonicated for 5-15 minutes at a temperature of 30°C. We varied our conditions more widely than this but included their experimental treatment also. Ordinarily, sonication was continued until there were no intact cells and both coarse and fine cellular debris were present. Sometimes the process was stopped when approximately 10% of the cells appeared morphologically intact but stained with trypan blue. In a few experiments, only very short times of sonication were desired. In still other experiments, sonication was prolonged until all cellular debris was uniformly fine.

Extracts prepared by the indirect sonication method for only 15 seconds to two minutes generally would give rise to weak positive transfer reactions (Table 3), but the activity resided in the centrifugal sediment and could be abolished by temporary suspension in chloroform-saturated Hanks or $HgCl_2$ solution. Hence, any transfer effect encountered with briefly sonicated cell suspensions can be considered due to the presence of competent living cells.

In one experiment designed to prevent damage that might be attributed to excessive exposure of liberated materials to sonic vibrations, competent peritoneal exudate cells from 4 donors (0.58ml) suspended in 4.5 ml G-S-C were sonicated in four stages in the Raytheon 200W, 10kc instrument. After 45 seconds, the supernatant was collected and kept at 0°C. and the sediment containing unbroken cells, removed by centrifugation, was resuspended and sonicated for another 45 seconds. The process was repeated two additional times. All four supernatant suspensions were injected intraperitoneally in a volume of 18 ml into one recipient. Thus cellular material set free was exposed to sonic excillations for only 45 seconds. The final residue which in the course of four sonications, had been exposed for three minutes was injected into another recipient. As is seen in Table 5 (PC1 473-A, recipients 6 and 7) and in Appendix Table VII-a, neither fraction yielded a transfer of hypersensitivity.

In an experiment designed to repeat the precise conditions for sonic disruption of cells as reported by Jeter, Tremaine and Seebohm (1954), competent cells were sonically disrupted in the 200W, 10kc Raytheon oscillator at a temperature of 30° ± 2°C, without encountering a positive transfer. All other sonic disruptions were performed at a chamber temperature of 0° to 4°C.

c. Numbers of Cells Transferred as Sonicates

As mentioned above, strong positive transfer reactions were obtained using donor:recipient ratios of 0.5:1 to 1:1 of living competent cells. For transfer of sonically disrupted cells, the amounts of starting cellular material has been usually four or more multiples of the amount which is effective in live cell transfers. Table 6 indicates that the amounts of cells transferred as sonic extracts have varied from donor:recipient

Table 3

TRANSFER OF CELLS SONICATED FOR BRIEF TIMES

Cell Source	Cell Pool	Recipient	Cells Used: Vol. (ml)	Cells Used: Count n x 10^8 (n)	Cells Used: D/R	Transferred Material	Preparation of Material	Route Given	T_2	T_3	T_4	T_5	T_6	Comment
Spleen	DnCl 280 (Percut)	6	0.53	----	6:1	Sonicate+ intact cells	2.5 min sonication in G-H. Most cells still intact	i.p.		++	++	tr	tr	Identical cell portions sonicated for 5 min was negative (recip. 7). This recipient had highest reaction to o-ClBCl of 6 sonic. recipients; viz. oClBCl-T_4 +; T_5 +; T_6 +.
Perit. Exudate	DnCl 283-A (Percut)	3	0.53	23.8	6:1	Sonic. Supernatant	1 min sonication in G-H	i.p.	tr	tr	±			Reactions not higher than toxicity controls; T_3, tr, tr, ±, ±,±: T_4, tr, ±, +.
		4				Sonic Sediment		i.p.	tr	tr	tr			
		5	0.53	23.8	6:1	Sonic. Supernatant + $CHCl_3$-treated Sediment	1 min sonication in G-H. Sediment held 20 min at 22° in $CHCl_3$-saturated G-H.	i.p.	prac 0	tr	tr			
Lymph Node	PCl 467-A	9	0.75	----	5:1	Sonicate+intact cells	1 min sonication in G-H	i.p.	tr	±	prac 0			ca. 30% cells appear normal, do not stain with trypan blue.Tbn transfer negative.
		8	0.75	----	5:1	Sonicate+intact cells	1.5 min sonication in G-H	i.p.	++	++	+			ca. 16% cells appear normal, do not stain with trypan blue.Tbn transfer negative.
		7	0.75	----	5:1	Sonicate+intact cells	5 min sonication in G-H	i.p.	tr	+	prac 0			Nearly all cells stain with trypan blue.
Spleen	PCl 472	11	0.72	----	3.3:1	Sonicate+intact cells	1 min sonication in G-S-C	i.p.	± (+++)	++ (+++)	±			ca. 25% of cells appear normal and do not stain with trypan blue.
Periton. Exudate	PCl 473-A	2	0.56	----	4:1	Sonic. Supernatant	0.75 min sonication in S-C	i.p.	0	0				
		3				Sonic. Sediment		i.p.	+	tr				Living Cells present. See recipients 4 and 5.
		4	0.56	----	4:1	Sonic Supernatant + $HgCl_2$-treated Sediment	0.75 min sonication in S-C Sediment held 2 hrs in 10^{-3} M $HgCl_2$ in saline.	i.p.	tr	tr				$HgCl_2$ lethal for living cells.
		5	0.56	----	4:1	Sonic. Supernatant + $CHCl_3$-treated Sediment	0.75 min sonication in S-C Sediment held 20 min in $CHCl_3$-saturated G-S-C	i.p.	prac 0	0				$ChCl_3$-saturated medium lethal for living cells.
		6	0.56	----	4:1	4 succesive Sonic. Supernatants	0.75 min sonication in S-C. Sediment re-sonicated another 45sec Repeated for 4 times. Supernatants pooled. Final Sediment used.	i.p.	prac 0	0				Supernatants subjected to 0.75 min sonication were negative.
		7				Final sediment after 4 successive sonications.		i.p.	0	0				Sediment sonicated 3 min was negative.

ratios of 1:1 to 11:1, an average being 6:1. Thus from 2 to 22 times as many cells as the amount of competent living cells required to produce a ' ++++ ' reaction, without the use of any chemical depilation, have been employed for transfer in the form of sonically disrupted cells. Likewise, recipients of sonic extracts were tested without the use of depilatory, excepting those described in III-C.

d. Time of Testing Recipients of Sonic Extracts

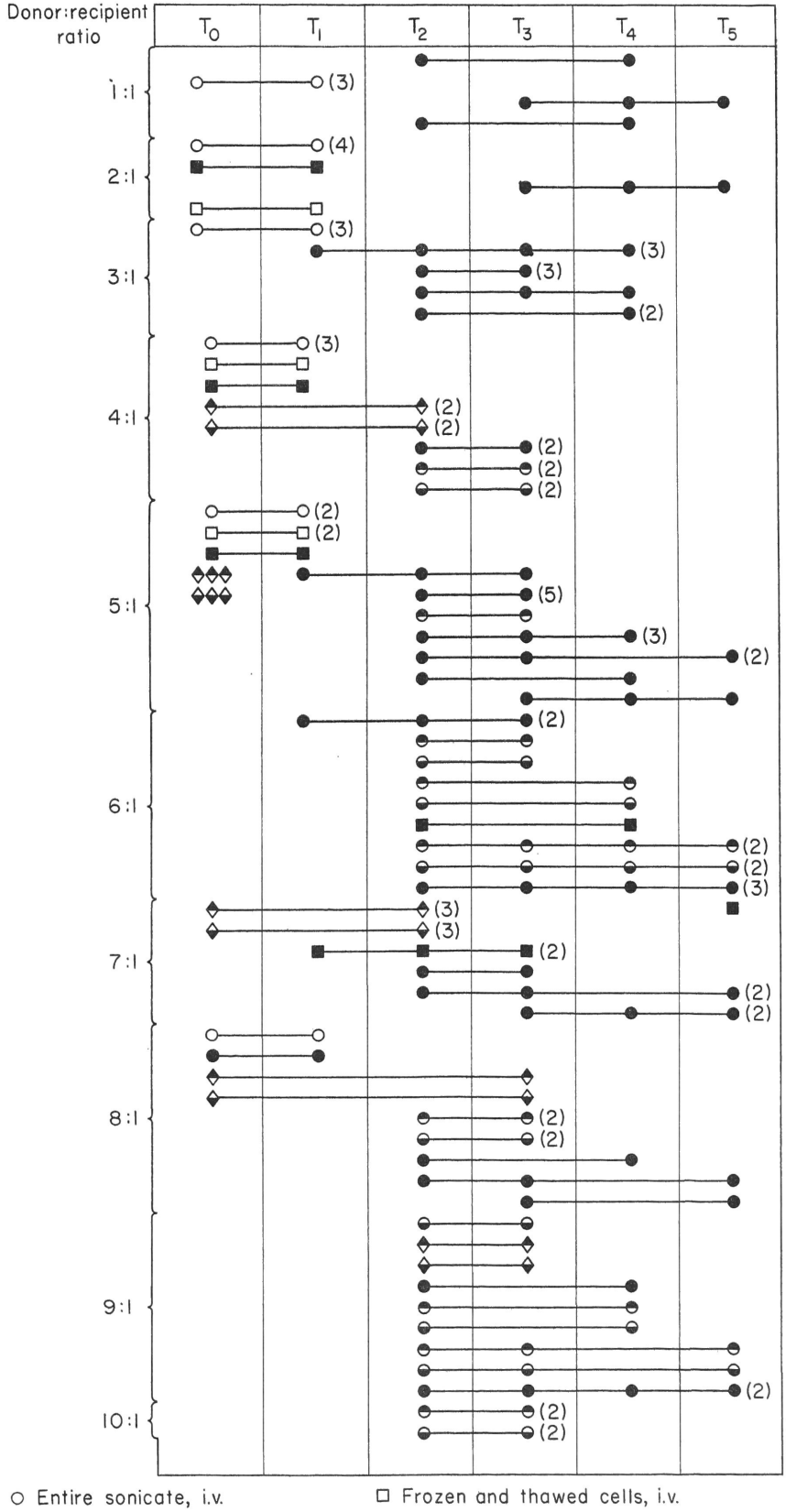
As seen in Table 5, tests were applied to recipients of extracts from 18 hours prior to transfer up to 5 days after transfer; generally, the first test was made two days (T_2) after the transfer when the material had been injected intraperitoneally, and 1 day (T_1) after the intravenous route had been used. Contact tests made 5 days or more after the first testing were apt to elicit anomalous positive reactions on control animals, indicating that active sensitization had occurred owing to exposure to allergen in the previous test. Consequently, re-testing of recipients of cellular materials had to be made within a narrow period of time following the initial test. Even reinjection of PPD can disclose active sensitization initiated by prior injections. Since recipients of living cells exhibit their hypersensitivity within the first few days after cell transfer, it was expected that animals receiving non-living material would respond at similar times. However, initial testing was withheld from a number of recipients for various times to allow for the possibility that recipients of cell extracts might require several days up to weeks to convert to a hypersensitive state. One example of this late testing may be cited, in which the first test was withheld until the twenty-first day after transfer, a time often used by Dr. H. S. Lawrence for testing after transfers of cells or cell extracts were made in man.

In Experiment PC1 477 (Appendix Table VII-b) peritoneal exudate cells and spleen cells harvested from 18 donors were subjected to sonication for 8 minutes by the indirect method at a temperature of 30°C (II-H). Half of each sonicate was injected directly into a recipient and the remaining half was separated into supernatant and sediment by centrifuging at 18,400 x g. for 15 minutes and injected into other recipients. Thus six recipients were available for testing. Contact tests were made on these animals in the first four days, but we refer, here, only to the tuberculin testing that was conducted (25μg PPD per 0.1ml) in the pattern and with the results shown in the following

Table 5

PATTERN OF TESTING RECIPIENTS OF DISRUPTED CELLULAR MATERIAL

(Animals receiving their first test later than five days not shown)



tabulation. It can be seen that no positive transfer was accomplished by means of any of the sonicated material.

Cells and Treatment	Tuberculin Tests (25μg PPD) T_1	T_2	T_{21}	T_{85}
Perit. Exudate, living (2:1)	+		+*	0
" " sonicate (9:1)			0	0
" " sonic. supernatant (9:1)		0	0	0
" " sonic. sediment (9:1)		0	0	0
Spleen Cells, living (3:1)	+		+*	0
" " sonicate (8.5:1)		0	0	0
" " sonic. supernatant (8.5:1)			0	
" " sonic. sediment (8.5:1)			0	

\+ signifies a positive reaction
0 signifies a negative reaction
* signifies the presence of an Arthus component

e. The Use of "Prepared" Recipients for Injection of Non-Living Materials.

In hopes of facilitating the transfer of hypersensitivity by non-living materials, animals used as recipients were sometimes treated prior to transfer to create peritoneal exudates, with the intention of depositing sonicated materials directly into the cellular exudates.

In another trial, viable normal cells were incubated in vitro with cellular extracts and the entire mixture was then injected into normal recipients. These experiments failed in establishing contact sensitivity in the respective recipients.

Two intended recipients received 1.5 ml of 12% sodium caseinate intraperitoneally two days prior to transfer. Sonically disrupted exudate cells (5.5:1) were injected intraperitoneally, and recipients were tested at appropriate times (Appendix Table VI).

Sonically disrupted peritoneal exudate cells from donors sensitive to picryl chloride (5.5:1; 0.83 ml) were incubated with peritoneal exudate cells from normal guinea pigs (5:1; 0.64 ml) at 37°C for 30 min. in gelatin-Hanks containing one-tenth part fresh guinea pig serum. The entire mixture was injected intraperitoneally into a normal recipient (Appendix Table VI).

Three recipients were primed with 0.5 mg of heat killed tubercle bacilli in 0.5 ml paraffin oil given intraperitoneally, and three weeks later a second intraperitoneal injection of 0.001 mg tubercle bacilli in saline was made in order to produce a predominantly mononuclear exudate. Two days later, such animals were used as recipients of cellular extracts or were reserved as toxicity controls, all being tested in parallel. The experiment failed, since all such "prepared" animals proved to be hyperreactive to contact testing with picryl chloride (Appendix Table I-a)

f. Results

Among 88 recipients of sonically disrupted cellular material, 71 failed to show any significant reactions, being either completely negative or showing only 'trace' irritations upon testing with the specific allergen (Table 6). Reactions slightly above this level were shown by 19 animals (reactions scored ' $\pm$ ' at one or more contact tests) for which an analysis is presented in Tables 8 and 3. Five recipients can be discounted as representing transfers effected with disrupted cells (Table 3) because the material was sonicated for such brief times that the presence of intact cells was noted at the time. An experiment (PC1 473-A, Appendix Table VII-a) specifically designed to determine whether cells could transfer sensitivity after very brief exposure to sonic oscillations demonstrated clearly that indeed this was possible, and that the positive reactions in recipients could be attributed to the live cells remaining in the sonicate since the activity resided in a low speed centrifugal sediment and was lost by treating the sediment with $CHCl_3$ - saturated buffer or with $HgCl_2$, or by sonicating for longer times.

When the criteria for positive transfer presented in Section III-A were applied to these 14 animals the following evaluation was obtained: In three cases the observed effect was that of active sensitization owing to previous testing, occurring both in recipients and toxicity controls.[1] In two other cases,[2] pretreatment of normal animals to give tuberculin-type peritoneal exudates made these animals hyperreactive to contact testing whether or not sonicated material was introduced. In

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[1] PC1 466, R 1, R 2; PC1 487-B, R 9 (details in Appendix Tables V-a and IX).

[2] DNC1 280, R 4Sp, R 8Sp (Appendix Table I-a).

five instances,[1] the same material or multiples thereof failed to produce any reactivity in other recipients injected at the same time. In four instances,[2] some of the normal animals serving as toxicity controls exhibited reactions of equal magnitude to recipients of sonically disrupted materials. In four cases,[3] recipients of sonicates showed low-grade reactivity to the specific allergen which was equalled or surpassed by the reactivity of the same animal to test with the non-specific test reagent, o-chlorobenzoyl chloride, and, of these, two animals possessed higher reactivity to both the specific and nonspecific compound than all other recipients in the same experiments, a fact which confirms the usefulness of o-chlorobenzoyl chloride in detecting non-specific reactivity; in the other two instances,[4] the highest reactivity of the corresponding toxicity controls was ' tr,' and the reactions of the two recipients were scored ' ± ', a level of reactivity not sufficiently different from the toxicity controls to be viewed as positive transfer.

There remains one animal, only, which may be regarded as an instance of possible transfer of hypersensitivity. This animal[5] exhibited at contact site T_1 a 24-hour reading of ' prac. 0 ' (like the toxicity controls), but a 48 hour reading of ' ± ' and a 72-hour reading of ' ~~++++~~ '. Unfortunately no controls could be obtained for this particular experiment and specificity was not determined. This must still remain an instance of questionable transfer since (a) an equal portion of the same cell pool sonicated for less time in the same medium minus cys. + RNA, was negative, and (b) tests on this with tuberculin remained negative. The observation on this recipient could not be repeated seven times using same medium, eight times using same medium without RNA.

In summary, of 88 recipients of sonically disrupted cells, there was only one recipient which could have represented an instance of possible transfer of contact hypersensitivity. This last finding could not be duplicated.

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[1] PC1 464, R5; PC1 487-A, R 2; PC1 487-B, R 9 (Appendix Tables IV-a, VIII-a, IX).

[2] DnC1 283-A, R 6, R 7; PC1 477, R 4 (Appendix Tables I-b, VII-b).

[3] DnC1 283-A, R 6, R 7; PC1 487-A, R 2, R 9 (Appendix Tables I-b, VIII-a).

[4] PC1 464, R 1; PC1 467-A, R 14 (Appendix Tables IV-a, VI).

[5] PC1 462, R 2; (Appendix Table III-b).

Table 6

SUMMARY OF ATTEMPTS TO EFFECT TRANSFER OF DELAYED-TYPE HYPERSENSITIVITY WITH SONICATED MATERIAL

Method of Sensitization	Cells Used	Suspending Medium	Sonication Technique	Time (min)	D/R Ratios per Recipient	Material Transferred †			Contact Reactions		Comment
						Entire	Supernatant	Sediment	Neg.	Not Neg.	
DnCl (Percutaneous) 280, 283-A	P.Ex	G-H	Indirect	6	9:1, 9:1	2			1	1	Unacceptable as transfer. See Table 8.
	"	"	"	4	6:1	1			1		
	"	"	"	4	6:1		1	1		2	Unacceptable as transfer. See Table 8.
	"	"	"	3	6:1	1			1		
	"	"	"	1	6:1, 6:1	1[a]	1	1	3		
	L.N.	"	Direct (50W)	10	9:1			1	2		
	"	"	" "	1	6:1	1			1		
	Spl.	"	Indirect	5	9:1, 9:1, 6:1	3			2	1	Unacceptable as transfer. See table 8.
	"	"	"	2.5	6:1	1				1	Unacceptable as transfer. See table 3.
DnCl Combination 283-B, 284-A	P.Ex.	"	Direct (50W)	10	9:1, 8.5:1, 8.5:1		3	3	6		
	L.N.	"	" "	10	11.5:1		1	1	2		
	"	"	" "	3	8:1	1			1		
	Spl.	"	" "	10	8:1	1			1		
	"	"	" (MSE)	5	4:1	1(iv)			1		
PCl Combination 467-A	P.Ex.	"	Indirect	4.5	5.5:1, 5.5:1	2			2		
	L.N.	"	"	5	5:1	1				1	Unacceptable as transfer. See Table 3.
"	"	"	"	1.5	5:1	1				1	Unacceptable as transfer. See Table 3.
"	"	"	"	1	5:1	1				1	Unacceptable as transfer. See Table 3.
487-A	P.Ex.	"	Direct (MSE)	7	5:1	1(iv)				1	Unacceptable as transfer. See Table 8.
"	"	"	" "	7	3:1, 1:1	2(iv)			2		
"	L.N.	"	" "	15	3.7:1, 3:1, 0.5:1	3(iv)			3		
"	"	"	" "	15	1.5:1	1(iv)				1	Unacceptable as transfer. See Table 8.
487-B	"	"	" "	9	4:1, 2:1, 1.8:1	3(iv)			3		
"	"	"	" "	4	ca. 5:1[b]		1[b]	1	2		
487-A	Spl.	"	" "	15	4.5:1, 3:1, 1.5:1	3(iv)			3		
487-B	"	"	" "	7	2:1, 2:1	2(iv)			2		
"	"	"	" "	11	ca. 8:1[c]	1(iv)			1		
462	P.Ex.	S-H+Cys	Indirect	5	6:1	1			1		
464	"	"	"	8	5.5:1	1				1	Unacceptable as transfer. See Table 8.
"	"	"	"	8	3:1, 2:1, 1:1	3			3		
463	L.N.	"	"	10	5.5:1	1			1		
464	"	"	"	6.5	6:1	1			1		
"	"	"	"	6.5	3:1	1				1	Unacceptable as transfer. See Table 8.
"	Spl.	"	"	5[d]	6.5:1	1			1		
463	P.Ex.	S-H+RNA+Cys	"	5	10:1		1[e]	1[e]	2		
"	"	"	"	5	4:1	1			1		
462	"	"	"	9	6:1	1				1	Possible transfer. See Table 8.
466	"	"	"	4	7.5:1	1				1	Unacceptable as transfer. See Table 8.
463	L.N.	"	"	10	7.5:1	1			1		
466	"	"	"	3	5:1	1			1		
463	Spl.	"	"	7[d]	6.5:1	1			1		
465	P.Ex.	G-H+RNA+Cys	"	5	7:1, 1.5:1	2			2		
"	L.N.	"	"	5.5	7:1	1			1		
"	Spl.	"	" (17°)	5	4.7:1	1			1		
"	"	"	" (40°)	5	2.3:1	1			1		
477	P.Ex.	G-H+RNA	" (30°)	8	9:1		1		1		
"	"	"	" (30°)	8	9:1			1		1	Unacceptable as transfer. See Table 8.
"	Spl.	"	" (30°)	8	8.5:1	1			1		
466	P.Ex.	H+Sucrose	"	3	5:1	1				1	Unacceptable as transfer. See Table 8.
"	Spl.	"	"	4.5	5:1	1			1		
467-A	"	S-H+PPD	"	4.5	6.5:1	1				1	Unacceptable as transfer. See Table 8.
473-A	P.Ex.	S-C	"	0.75	4:1		1		1		
"	"	"	"	0.75	4:1			1		1	Unacceptable as transfer. See Table 3.
"	"	"	"	0.75	4:1, 4:1	2[a]			2		
"	"	"	"	4x0.75	4:1		1[f]	1	2		
472	L.N.	G-S-C	"	4.5	5:1	1			1		
"	Spl.	"	"	5	3.3:1, 3.3:1	2			2		
"	"	"	"	4.5	3.3:1	1			1		
"	"	"	"	1	3.3:1	1				1	Unacceptable as transfer. See Table 3.

Legend appears below Table 7.

Table 7

SUMMARY OF ATTEMPTS TO EFFECT TRANSFER OF DELAYED-TYPE HYPERSENSITIVITY WITH CELL HOMOGENATES AND FROZEN AND THAWED CELLS

A. Cell Homogenates and Cell Fractions

Donor Pool Combination Method	Cell Source	Medium	D/R Ratios per Transfer	Material Transferred			Contact Reactions		Comments
				Entire	Cell Sap (i.v.)	Microsomes (i.p.)	Neg.	Not Neg.	
DnCl 284	Spleen	1% G-H	9:1	1[g],1[h]			2		
PCl 491-A	L.N. Slices +Spleen wbc	Askonas	8:1[i], 4:1, 4:1		2	3	5		
" "	"	Askonas	4:1		1			1	Unacceptable as transfer. See Table 9.
PCl 492-A	"	Askonas-Hanks (Turk)	5.4:1, 5.4:1		2	2	4		
" "	"	"	ca. 3:1[j]		1		1		
" "	"	"	5.4:1		1	1		2	Unacceptable as transfer. See Table 9.
PCl 492-B	"	Askonas	6.7:1, 6.7:1, 6.7:1		1	3	4		
" "	"	Askonas			1	1[k]	2		
" "	"	Askonas	6.7:1		2			2	Unacceptable as transfer. See Table 9.

B. Frozen and thawed Cells

Donor Pool Combination Method	Cell Source	Medium	D/R Ratios per Transfer	Route of injection	Contact Reactions		Comments
					Neg.	Not Neg.	
PCl 487-A	P.Ex.	G-H + DNase (0.1mg/ml)	4.5:1	i.v.	1		
PCl 516/Tb 137	"	" " "	6:1	i.p.	1		
PCl 487-A	L.N.	" " "	5:1	i.v.		1	Unacceptable as transfer. See Table 9.
PCl 487-B	"	" " "	5:1	i.p.	1		
" "	"	" " "	2:1	i.p.		1	Unacceptable as transfer. See Table 9.
PCl 516/Tb 137	"	" " "	6:1, 6:1	i.p.	2		
PCl 487-A	Spleen	" " "	5:1	i.v.	1		
PCl 487-B	"	" " "	4:1	i.p.	1		
" "	"	" " "	2:1	i.p.		1	Unacceptable as transfer. See Table 9.
PCl 516/Tb 137	"	G-H + DNase (0.25mg/ml)	9:1	i.p.	1		
PCl 462	"	S-H+Cys+$MgSO_4$+DNase (0.2mg/ml)	7:1	i.p.	1		
" "	"	S-C +NaF	7:1	i.p.	1		
PCl 463	"	S-H+RNA+Cys[l]	6.5:1	i.p.	1		
PCl 464	"	S-H + Cys[m]	5.6:1	i.p.		1	Unacceptable as transfer. See Table 9.

Legend for Tables 6 and 7

† - Transfers effected by the intraperitoneal route, unless otherwise indicated.

a - Supernatant, plus sediment treated to kill living cells, injected into same recipient.

b - Sonicated lymph node residue after free cells were removed; supernatant injected i.v. & i.p.

c - Resonicated stromata.

d - Held overnight at 4°C.

e - 15,000rpm-1.25 hours, producing cell sap (supernatant), and cell particulates.

f - Pooled supernatants from 4 successive 0.75 min sonications.

g - Low speed (1500rpm-15') centrifugation supernatant injected i.p.

h - Sediment from (a) injected i.p.

i - D/R ratio as the combined amount for lymph node + spleen cells.

j - Cell sap from rehomogenized stroma.

k - Cell sap and final residue from rehomogenized pellet.

l - Held 3 days at -20°C. Frozen and thawed only 1x.

m - Held 19 hours at -68°C. Frozen and thawed only 2x.

Table 8

ANALYSIS OF SLIGHT REACTIONS ENCOUNTERED IN CERTAIN RECIPIENTS OF SONICALLY DISRUPTED MATERIAL

Cell Source	Cell Pool	Recip-ient	Cells Used: Vol. (ml)	Cells Used: Count $n \times 10^8$ (n)	Cells Used: D/R Ratio	Transferred Material	Preparation of Material	Route Given	Contact Reactions: T_o	T_1	T_2	T_3	T_4	T_5	Comment
							PART I: SONICATION								
P.O.	DNCl 280	4-Sp*	1.35	---	9:1	Sonicate	Sonicated 6 min. in G-H.	i.p.			+±	+±	++	tr	Transfer of the same material into normal recipients (R 3, R 7) was without effect. Special TC (TC2, TC 4), pre-treated in identical fashion, reacted as strongly (T_3 +±; T_4 ++).
SPL.	"	(8-Sp)*	0.80	---	9:1	"	Sonicated 6 min. in G-H.	i.p.				+±	+±	tr	
P.O.	DNCl 283-A	6	0.53	23.8	6:1	Sonic.Supernatant	Sonicated 4 min. in G-H, (2000 rpm 15')	i.p.			0	+±	+		This reactivity is not significant: TC reactions were unusually high in this expt. (T_3: tr, tr, ±, ±, ±; T_4: tr, ± +). In addition, both recipients reacted as strongly to oClBCl (T_3 ± and +±; T_4 + and ±).
	"	7	"	"	"	Sonic. Sediment		"			tr	±	±		
P.O.	PCl 462	2	0.55	---	6:1	Sonicate	Sonicated 9 min. in S-H-RNA-Cys.	i.p.		0 (±)† [+++]†	+++ (++++)	++++			An equal portion of cells sonicated in 35% S-H produced no reactivity in R 3. Tbn reactions at T_2 and T_3 were negative. No TC could be included in this expt. and no oClBCl test was made to determine whether the reactivity of this recipient was specific. The conditions of this expt. were repeated 8 times, and 7 additional attempts were made using S-H+Cys with no comparable result.
P.O.	PCl 464	1	0.59	---	5.5:1	Sonicate	Sonicated 8 min. in S-H-Cys.	i.p.			tr (±)	tr			This level of reactivity is not significant: Although reactions on three TC animals were less, centrifugal fractions of the same sonicate produced no effect in R 6, 7, 8. No test for specificity was made.
L.N.	"	5	0.36	---	3:1	"	Sonicated 6.5 min. in S-H-Cys	"			± (+)	tr			
P.O.	PCl 466	1	057	---	7.5:1	Sonicate	Sonicated 4 min. in S-H-RNA-Cys.	i.p.			tr	0		+p **	These T_5 reactions are manifestations of active sensitization by the previous testing. One of 3 TC was sensitized by T_5(+±); all reacted strongly by T_6 (+±, +±, +++).
SPL.	"	11	0.89	---	5:1	"	Sonicated 4.5 min. in H+Sucrose	"			tr	tr		+	
SPL.	PCl 467-A	14	1.8	---	6.5:1	Sonicate	Sonicated 4.5 min. in S-H + PPD (10% fresh serum, 40% aged serum, 2.5 γ PPD/ml)	i.p.			0	±	0		The level of reactivity is not significant: TC at T_2 were tr, tr, tr, tr. The Tbn reaction of this recipient was negative.
P.O.	PCl 477	4	1.26	37.5	9:1	Sonic.sediment,washed	Sonicated 8 min. in G-H+RNA(30°)	i.p.			±		tr		One of 4 TC reacted as strongly at T_3 (T_2 0(+p); T_4 ±)
P.O.	PCl 487-A	2	0.49	8.4	5:1	Sonicate	Sonicated 7 min. in G-H (MSE)	i.v.	±	tr					There is as high a reaction to oClBCl on this recipient at T_2 (±). Transfers of the same material in 3:1 and 1:1 ratios were negative in R 3, and 4.
L.N.	PCl 487-A	9	0.16	9.5	1.5:1	Sonicate	Sonicated 15 min. in G-H (MSE)	i.v.	±	prac 0					Transfers of 2x the same material (3.7:1, 3:1) in R 7, 8, were ineffective. There was as high a reaction to oClBCl at T_o (±).
L.N.	PCl 487-B	9	0.28	9.2	1.8:1	Sonicate	Sonicated 9 min. in G-H (MSE)	i.v.	tr	tr					The T_4 reaction is due to active sensitization by prior testing as is seen in 3/4 TC reactions at T_4 (o, ±, +, +). Transfer of 2x same material into R 8 was without effect.

* - Special recipients, were pre-treated to have a peritoneal exudate present at the time of transfer. See Appendix Table I-a.

** - p following a contact reading indicates spots and patches at the reaction site; not confluent reactions.

† - ( ) shows reading at same test site, one day later: [ ] shows reading at that site two days later.

Table 9

ANALYSIS OF SLIGHT REACTIONS ENCOUNTERED IN CERTAIN RECIPIENTS OF CELL HOMOGENATES AND FROZEN AND THAWED CELLS

Cell Source	Cell Pool	Recipient	Cells Used: Vol. (ml)	Cells Used: Count $\underline{n} \times 10^8$ (n)	Cells Used: D/R	Transferred Material	Preparation of Material	Route Given	Contact Tests: T_o	T_1	T_2	T_3	Comment
A. Cell Homogenates and Cell Fractions													
L.N. & SPL.(wbc)	PCl 491-A	6			4:1	Cell Sap	Homogenized in Askonas, 5000 x g sediment discarded. (100,000 x g. -60') for cell sap, pellet.	i.v. & i.p.	± (±)				Transfers of 2x same material into R.4 and the same amount into R.8 were without effect. This animal had the highest reaction to oClBCl at T_o(±) of 6 recipients of cell fractions.
L.N. & SPL.(wbc)	PCl 492-A	6			5.4:1	Cell Sap	Homogenized in Askonas-Hanks (Turk), sediment at 5000 rpm 15' rehomogenized. Supernatant (100,000 x g.-60') gave cell sap and microsomal pellet.	i.v.	tr (+)				This reactivity is not significant: TC controls reacted as strongly or more so at T_o (tr,tr, tr(±), ±, +, ±, ±). In addition, R.11 had the highest T_o reaction to oClBCl of 7 recipients of cell fractions (±).
"	PCl 492-A	11			5.4:1	Microsomes only		i.p.	± (±)				
L.N. & SPL.(wbc)	PCl 492-B	3			6.7:1	Cell Sap	Homogenized in Askonas Medium Sediment of 5000rpm-15' re-homogenized. Supernatant (100,000 xg.-60') gave cell sap and pellet	i.v.	tr				This reactivity is not significant: 3 TC of 6 reacted as strongly at T_2 (0,0, tr, ±p, +, +). In addition, R.2 was equally reactive to T-1/4 oClBCl
									T-1/4	T_o			
"		2			6.7:1	Cell Sap		i.v.	tr (±)				
B. Frozen and Thawed Cells									T_o	T_1	T_2	T_3	
SPL.	PCl 464	11	0.63	---	5.6:1	Frozen & thawed cells	Held in 10% S-H-Cys 19 hrs in dry ice, then frozen & thawed 2x	i.p.			±	0	This reactivity is too low to be significant, although none of the three TC has as strong T_2 reactions.
L.N.	PCl 487-A	11	0.55	32.5	5:1	Frozen & Thawed Cells	Frozen in G-H DNase(0.1 mg/ml) then frozen & thawed 10x.	i.v.	±	±p			This recipient reacted equally strongly to oClBCl.
SPL.	PCl 487-B	5	0.49	9.5	2:1	Frozen & Thawed Cells	Frozen in G-H + DNase(0.1 mg/ml) then frozen & thawed 7x.	i.v.	tr (±)	tr		++	Transfer of 2x same material was inaffective in R.4. TC animals were becoming actively sensitized by T_4 (See Comment on PCl 487-B, R. 9)
L.N.	PCl 487-B	11	0.31	10.2	2:1	Frozen & Thawed Cells	Frozen in G-H + DNase(0.1 mg/ml) then frozen & thawed 7x.	i.p.	±	0		tr	Recipient 10 given 2.5x same material showed no reactivity.

** - p following a contact reading indicates spots and patches at the reaction site; not confluent reactions.

† - ( ) shows reading at same test site, one day later; [ ] shows reading at that site two days later.

3. Transfer of Frozen and Thawed Cells

In all, 19 transfers were made using cells disrupted by multiple freezings and thawings, fifteen of these appearing in Table 7. In thirteen, desoxyribonuclease was added to facilitate injection by reducing the viscosity; only one-quarter of the amount used routinely by Dr. H. S. Lawrence was employed. Ratios used for transfer varied from 2:1 to 10:1 and such transfers were generally made by the intraperitoneal route (one was made intravenously, and four intradermally in multiple sites). Times of testing are shown in Table 5. Eleven recipients had contact reactions of trace level or less, and four had reactions scored as ' ± ' (Table 9). Of these, two[1] are considered to be spurious by virtue of the fact that multiples of the same material injected into other normal recipients failed to cause any reactivity at all; another animal reacted[2] equally strongly to oC1BC1 and in still another case[3], although the recipient reacted higher than the three corresponding toxicity controls at test T_2, the ' ± ' reaction can hardly be accepted as a positive transfer. One of the animals mentioned above as receiving frozen and thawed cells intradermally (3.3:1 transfer with lymph node cells) was tested only with tuberculin at test T_2 and was negative, although living peritoneal exudate and spleen cells of the same donors resulted in definite tuberculin reactivity.

Three other recipients in the same experiment (PC1 477, Appendix Table VII-b) received frozen and thawed cells intradermally and were not tested with tuberculin until specified times had elapsed, one after 28 days, another after 43 days, and the third after 69 days. None of the animals reacted to tuberculin testing.

> In an experiment not described elsewhere, peritoneal exudate cells (10:1, 1.09ml) from "Combination Method" donors were frozen and thawed inside a dialysis sac in 15ml S-H. After dialysis for 2 days against saline and water, the cell residue inside the sac and the lyophilized dialysate were injected intradermally into separate recipients. Two other recipients received lymph node cells (10:1 5.5ml) treated in identical fashion, except that DNase was added to the freezing medium. Contact and tuberculin tests made at T_2 were ' 0 ' in all cases.

There was, therefore, no instance of reactivity which would suggest transfer of contact or tuberculin hypersensitivity by means of frozen and thawed cells.

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[1] PC1 487-B, R 5, R 11 (Appendix Table IX).

[2] PC1 487-A, R 11 (Appendix Table VIII-a).

[3] PC1 464, R 11 (Appendix Table IV-a).

4. Transfer of Homogenates and Cell Fractions

Cells were subjected to homogenization under hypertonic conditions with the purpose of maintaining subcellular particles morphologically intact and biochemically active. Most homogenates were made in 0.35M sucrose and were subjected to ultracentrifugation to obtain microsomal and cell sap fractions, which were reported by Turk to be able to transfer picryl chloride hypersensitivity.

a. Media for Homogenization

As outlined in Table 7, competent cells were homogenized in three different types of medium.

i). 1% Gelatin-Hanks: Gelatin was added to 1% concentration to increase markedly the viscosity of Hanks solution and hence the shearing force during homogenization. Transfer of a low-speed centrifugal sediment (1300 rpm, 5 minutes), and of the corresponding supernatant separately, was carried out using spleens from animals sensitized by the "Combination Method".

ii). Askonas' medium: Fourteen transfers were performed with homogenates prepared in this buffered sucrose medium (Askonas, 1961) which was designed to obtain from plasma cells microsomes which retained their ability to incorporate C^{14}-amino acids into protein. The lymph nodes and spleens were homogenized and transferred separately.

iii). The medium of Turk: The medium actually used by Turk (personal communication) in his claim of positive transfer of contact hypersensitivity (1961) was a mixture of the Askonas sucrose medium (low Mg ++, low electrolyte concentration) with Hanks solution (high in electrolytes, with both Mg ++ and Ca ++). In seven transfers made with this system in a large experiment, lymph node slices and spleen white cells were together suspended in four volumes of Hanks solution and made 0.35 M in sucrose by the addition of one volume of five-times concentrated Askonas medium. They were homogenized at 4°C (II-J). Microsomal and cell sap fractions were obtained by ultracentrifugation in the Spinco Model L preparative centrifuge (100,000 x g for 60 minutes).

As shown in Table 7, ratios of 4:1 to 8:1 were transferred and

the recipients were tested variously, from within one hour after transfer up to 2 days afterwards (Table 5).

b. Results

Homogenates made of validated, competent transfer cells were injected into 21 recipients, among which only four recipients had reactions above trace level (Tables 7 and 9). In three out of these four[1] reactions to the specific allergen were no higher than those of the toxicity controls which, it must be noted, were unusually high in Experiments PC1 492 A and B for reasons which are not clear. In one instance,[2] transfer of double the amount of the same cell sap failed to effect any reactivity in another recipient. In three of the animals[3] reacting to the level of ' $\pm$ ' or above to the specific allergen, picryl chloride, reactions equal to or greater than these were found in parallel testing with 15% oClBCl, hence these slight reactions can be dismissed as being non-specific in nature. None of 21 recipients suggested that contact hypersensitivity had been transferred by cell homogenates, or by cell sap and microsomal fractions obtained therefrom.

C. The Hazard of Chemical Depilation of Test Sites:

It is our practice to test animals on skin sites from which the hair has been removed by an electric clipper, taking great care to prevent irritation of the skin during the clipping. In the work of Jeter et al. (1954) and Turk (1961) in which positive transfer of contact hypersensitivity is claimed to be effected in guinea pigs by means of cell extracts, skin sites were treated with chemical depilatories. In the experiments of Jeter and co-workers, the sites were treated for five minutes with the depilatory "Nair" (thioglycollate, the active ingredient) at times from 24 to 4 hours prior to testing. Previous experiences in this laboratory had cast strong doubt as to the advisability of removing hair stubble by chemical means owing to hyperreactivity which results from the treatment. As Dr. Peyton Rous (personal communication) found in rabbits, chemical depilation initiates intense epithelial hyperplasia.

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[1] PC1 492-A R6, R11 (Appendix Table X-a); PC1 492-B R2 (Appendix Table X-b)

[2] PC1 491-A R6 (Appendix Table VIII-b)

[3] PC1 491-A R6, PC1 492-A R6, PC1 492-B R2 (Appendix Tables X-a, X-b).

To determine the effect of chemical pre-depilation in the manner used in Dr. Jeter's laboratory, animals were tested with the specific allergen both on a clipped site and on a pre-depilated site. In addition, in order to be able to detect any non-specific irritant effect of the depilatory, the chemically unrelated compound, o-chlorobenzoyl chloride, used at slightly irritating concentrations, was also painted on clipped and on pre-depilated sites on the same animals. (In IV-C, the use of o-chlorobenzoyl chloride as a non-specific test reagent is discussed.)

> The method of depilation of the sites was as follows: The area to be tested, clipped as usual with the electric clipper, was ringed with vaseline to prevent retention of any depilatory at the hair roots. The depilatory, "Nair", was applied gently with a wooden tongue depressor. After exactly five minutes, the depilatory was removed with moist cotton sponges and the area was dried with a linen towel and treated with talcum to prevent chafing.

The depilated skin site 24 hours after depilation had the following peculiar characteristics. The skin was brittle to the touch, tufts of hair grew back irregularly, often there were obvious signs of irritation in the form of tiny raised papules or rather erythematous patches. Whenever an animal succeeded in scratching a depilated site there was a pronounced edema and erythema which has rarely been seen on normal clipped skin . The results to be presented below will indicate that chemical depilation of the skin serves to exacerbate any injury or irritation to that site. (Contact testing was usually performed on sites depilated 24 hours in advance, but no difference was observed in PCl 477 (Appendix Table VII-b) when the interval was reduced to four hours).

When recipients of competent living cells destined to show positive reactions were tested on a pre-depilated site and, at the same time, on a non-depilated clipped site, it was found that the positive reactions on the pre-depilated sites were scored as higher reactions than those on the clipped sites.[1] On the other hand, when recipients of living cells which showed weakly positive or questionable reactions on simple clipped sites were tested simultaneously with the specific allergen on pre-depilated sites, often reactions occurred which were stronger and would be visually rated as positive reactions.[2]

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[1] For example, see DnCl 283-B, R 1, R 6 (Appendix Table II-a)

[2] For example, see DnCl 283-A, R 13; DnCl 284-A, R 2, R 8, R 11 (Appendix Tables I-b and II-a).

Table 10

THE EFFECTS OF CHEMICAL DEPILATION OF SKIN SITES BEFORE AND AFTER CONTACT TESTING

Part A: Irritative Reactions to Contact Test (± or greater)

	DnCl or PCl		oClBCl	
Animals	Normal Skin	Pre-depil. Skin*	Normal Skin	Pre-depil. Skin*
Toxicity Controls	4/21(19%)	16/21(77%)†	2/10(20%)	5/10(50%)
Sonicate Recipients	5/37(15%)	22/37(59%)††	6/25(24%)	13/25(52%)

Part B: Instances of Irritative Reactions at Pre-depilated Sites Greater Than at Corresponding Normal Sites

Animals	DnCl or PCl	oClBCl
Toxicity Controls	14/21(67%)	6/12(50%)
Sonicate Recipients	22/37(60%)	11/26(42%)

Part C: Instances of Irritative Reactions at Post-Depilated Half-Sites Greater than at Corresponding Normal Half-Sites.

Animals	PCl	oClBCl
Toxicity Controls	4/7(57%)	1/2(50%)
Cell Fraction Recipients	7/11(63%)	4/4(100%)
Live Cell Recipients	4/5(80%)	---

* Pre-depilation made 24 hours before application of contact test in most experiments. The time was altered in PCl 477 (Appendix Table VII-b) to 4 hours before contact testing. Since the result was consonant these animals are included in the table.

† Of the 16, 10 (62%) gave reactions at 24 or 48 hours higher than ±.

†† Of the 22, 13 (59%) gave reactions at 24 or 48 hours higher than ±.

Among reactions observed to the specific allergen on recipients of living cells, 16/18 produced were greater on pre-depilated skin than on normal clipped skin.

The question arises, then, whether the pre-depilation serves usefully to magnify weak positive reactions. To test this, normal animals were tested likewise on simple clipped skin sites and on pre-depilated sites with both picryl chloride or dinitrochlorobenzene in sub-toxic concentration, and with o-chlorobenzoyl chloride, at a concentration approaching the limit of toxicity or normal skin. These normal animals were toxicity controls in transfer experiments and the results of the contact testing are presented in Table 10. In 14 out of 21 instances (Table 10, Part B), the reactions to the "specific" test allergen (PCl or DnCl) were stronger on the pre-depilated site than on the clipped skin site.[1] As is seen in Table 10, Part A, only four out of 21 tests on the clipped sites could be rated as high as ' ± ', whereas on the pre-depilated sites on 21 animals, six readings were scored ' ± ' and ten were greater and would have been considered as definitely positive although not strongly so. This exaggerated irritability of the pre-depilated sites on normal animals, furthermore, was observed also with the oClBCl in six out of twelve instances[2]. There can be no question that the effect of the predipilation had been to exacerbate non-specific irritation to the skin, for these animals were normal and no component of hypersensitivity could have been present in any of the reactions cited. It may also be added that there was no case in which the toxicity of the test substance used was greater on a simple clipped site than on a pre-depilated one.

It thus becomes clear that it would be a mistake to conclude that pre-depilation could be used in an unambiguous way to magnify specific hypersensitivity reactions, since it has been shown that such treatment would exaggerate any non-specific inflammatory process. As to the reason why the recipients which show positive reactions after transfer of live cells give stronger reactions to specific allergen on pre-depilated sites, the possibility exists that the hypersensitivity-mediated component which initiates the reaction has not simply been magnified, but rather that the inflammatory process which

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[1] For example, See PCl 477, TC 3, TC 4; DnCl 283-A, TC 1, TC 2, TC 3; DnCl 284-A, TC 1, TC 2, TC 3 (Appendix Tables VII-b, I-b, and II-b).

[2] For example, See DnCl 283-A, TC 2; DnCl 283-B, TC 2; DnCl 284-A, TC 1, TC 2, TC 3 (Appendix Tables I-b, II-a, and II-b).

follows the initial hypersensitivity reaction in the skin is exaggerated. It is difficult to know precisely the complex results of hyperplasia which are initiated by the use of chemical depilatories.

Bearing these considerations in mind, we may proceed to examine reactions on recipients of subcellular materials with respect to normal and predepilated test sites. Of 37 tests to the specific allergen on recipients of cellular extracts made simultaneously on pre-treated and normal clipped skin, 22 reactions on depilated sites were greater than on the corresponding clipped sites (Table 10, Part B), and in only one instance was the situation reversed. Of these 37 tests, only five readings[1] on clipped sites could be scored as high as ' $\pm$ ' (Table 10, Part B). On the pre-depilated sites, 13 out of 22 were still higher and could have been considered positive or questionably positive reactions[2], were it not for the fact that 10 out of 16 of the toxicity controls (an equal percentage) showed parallel reactivity on pre-depilated sites; hence they cannot be considered as manifestations of true hypersensitivity. The reactions to oClBCl on cell extract recipients were greater in 11 of 26 instances on depilated skin sites (Table 10, Part B), again in general agreement with observations on toxicity controls.

The use of chemical depilatory in reading contact reactions also offers hazards. As was mentioned above, in his report of transfer of contact hypersensitivity by means of cell fractions, Turk (1961) depilated the test sites before reading at 24 hours by means of a barium sulfide paste. The sites were re-read at 48 hours and the highest of the two readings was published. Almost invariably the 48 hour readings had been greater than those at 24 hours (Turk, personal communication).

In order to ascertain whether "post-depilation" causes non-specific reactivity test sites made on clipped skin were depilated at 24 hours but only over one-half of the entire test area (the other half was protected by making a line with vaseline through the middle of the site) during 5 minutes application of the depilatory "Nair" as above. Recipients of living cells, recipients of cellular homogenates and normal animals were tested in this way.

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[1] DnCl 283-A, R 7; PCl 477, R 4 (Appendix Tables I-b and VII-b).

[2] For example, see DnCl 283-A, R 3, R 4, R 7, R 9, R 12; DnCl 283-B, R 7; DnCl 284-A, R 5, R 6, R 12; PCl 477, R 3, R 4 (Appendix Tables I-b, II-a, II-b, VII-b).

Both halves of the test sites were read individually on all animals 24 hours after depilating, that is, 48 hours after testing (Table 10, Part C).

The exacerbation of the reactions on positive living cell transfers was similar to that reported for pre-depilated sites and was seen in four out of five such recipients treated in this way.[1] Of the "toxicity controls", four out of seven had greater reactions to picryl chloride on the post-depilated half of the sites than on the undepilated.[2] (Of two oClBCl sites treated in this fashion on control animals, one was stronger on a pre-depilated half.) Of eleven recipients of microsomal or cell sap fractions of homogenized competent cells, seven had greater reactions on the depilated half of the sites than on the untreated half, of which, again, all might be construed as questionable positive reactions, and were like the reactions on the controls.[3] Of post-depilated oClBCl test sites on four extract recipients, all four had higher readings than on the untreated half.[4] Therefore, still further evidence accrues emphasizing the hazard of using chemical depilatories for this purpose.

D. Attempts to Release "Transfer Factor" by Exposure of Cells to the Specific Antigen.

It was reported by Lawrence and Pappenheimer (1957) that when cells from tuberculin-sensitive human donors were incubated with PPD in vitro, the cells lost their capacity to transfer tuberculin hypersensitivity, and at the same time there was released into the menstruum containing the tuberculin a "transfer factor" which could sensitize human recipients to tuberculin. Later it was claimed that this effect is specific, for when cells of two competencies (diphtheria toxoid and tuberculin hypersensitivity) were incubated with PPD, the cells lost only their capacity to transfer tuberculin hypersensitivity (Lawrence, 1959). The theoretical importance of these results, if they truly represent a general pricipal, is great; consequently experiments based on the experimental conditions of the human experiments were carried out in the guinea

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[1] PCl 491-A, R 2; PCl 492-A, R 1, R 2, R 3, R 4 (Appendix Tables VIII-b, IX-a).

[2] PCl 491-A, TC 1; PCl 492-A, TC 1, TC 2, TC 4, TC 5 (loc. cit.)

[3] PCl 491-A, R 4, R 5, R 6, R 7; PCl 492-A, R 5, R 6, R 8, R 10, R 11 (loc. cit.)

[4] PCl 491-A, R 4, R 5, R 6, R 7 (Appendix Table VIII-b).

pig system.

1. Peritoneal exudate cells and lymph node cells were taken from donor guinea pigs which had been injected 17 days previously with 1.5mg heat killed tubercle bacilli (Jamaica No 22) in paraffin oil and vaseline into the nuchal muscles (Appendix Table XI). The donors were not subjected to skin tests. One aliquot of the pool of peritoneal exudate cells from 18 donors was used to validate the competency of the untreated cells. The cells were suspended in 12ml culture medium and incubated for two hours at 37°. The medium was Eagle's medium containing amino acids doubled in amount, vitamins in quadrupled amount, sodium pyruvate, ferric nitrate, increased bicarbonate and 5% newborn calf serum (Gomatos et al., 1962). The cells were then washed in gelatin-Hanks and injected intraperitoneally into normal recipients. The clear positive reactions of Recipient 1 at T_2 and T_3 indicated that the cells were fully competent. A similar aliquot was incubated as above except that special excipient-free PPD was included in the medium at a concentration of 3.3µg/ml. After the incubation the cells were sedimented, washed in gelatin-Hanks and injected into one recipient, while the supernatant, after being clarified at 9000rpm for 10min, was injected by the intraperitoneal route into another. Tests at T_2 and T_3 with the same excipient-free PPD (25µg/0.1ml) showed that this portion of the cells was highly competent and did not disclose any diminution in the ability to transfer because the cells had been incubated with PPD (Recipient 2), nor any ability of the supernatant to effect transfer (Recipient 3). The third aliquot of cells was suspended in 14 ml of the serum-Eagle's medium containing Parke-Davis and Co, Old Tuberculin (dialyzed and used in a concentration of 1:200) and incubated for three hours at 37° inside a sterile dialysis sac against 42ml of the serum-Eagle's medium. The cells, washed with gelatin-Hanks, remained fully competent to transfer tuberculin hypersensitivity (Recipient 4), the fluid immediately over the cells and containing the tuberculin failed to transfer, and the outer dialysate, which would be expected at equilibrium to contain three fourths of all dialyzable substances free of O.T. failed to effect transfer.

2. In the same experiment, lymph node cells were incubated as follows: one-half (D/R ratio of 9:1. 0.31ml packed cells, 10.3×10^8 cells) was incubated for two hours at 37° in 12 ml of the same fortified serum-Eagle's and the other half was incubated in the same medium containing dialyzed Old Tuberculin (1:200). The results of tests with 25μg PPD at T_2 and T_3 clearly show that the cells incubated with 0.6. transferred without diminution and that the supernatant of the incubation mixture was unable to transfer any sensitivity.

3. Incubation of competent cells with specific antigen in the presence of fresh guinea pig serum containing active components of serum Complement gave the same results (PC1 467, Appendix Table VI). Splenic cells from 6.5 donors (cell volume, 1.80ml) were incubated for 1.75 hours at 37° in 10ml 50% guinea pig serum-Hanks (1.5 of the serum was freshly drawn) containing 25μg PPD. The cells retained their competence to transfer both picryl chloride and tuberculin hypersensitivity, (Recipient 12) whereas the culture supernatant (6ml out of 10ml), was unable to effect transfer as determined by testing with 50μg PPD at T_2 and T_3 (Recipient 13).

The results of these experiments indicate that competent guinea pigs cells do not suffer any diminution in their capacity to transfer tuberculin hypersensitivity upon exposure in vitro to PPD or Old Tuberculin, and that no factor is released into the medium which has a capacity to do so.

IV

ATTEMPTS AT UNDERSTANDING THE BASIS OF CONTACT REACTIVITY

IV. Attempts at Understanding the Basis of Contact Reactivity

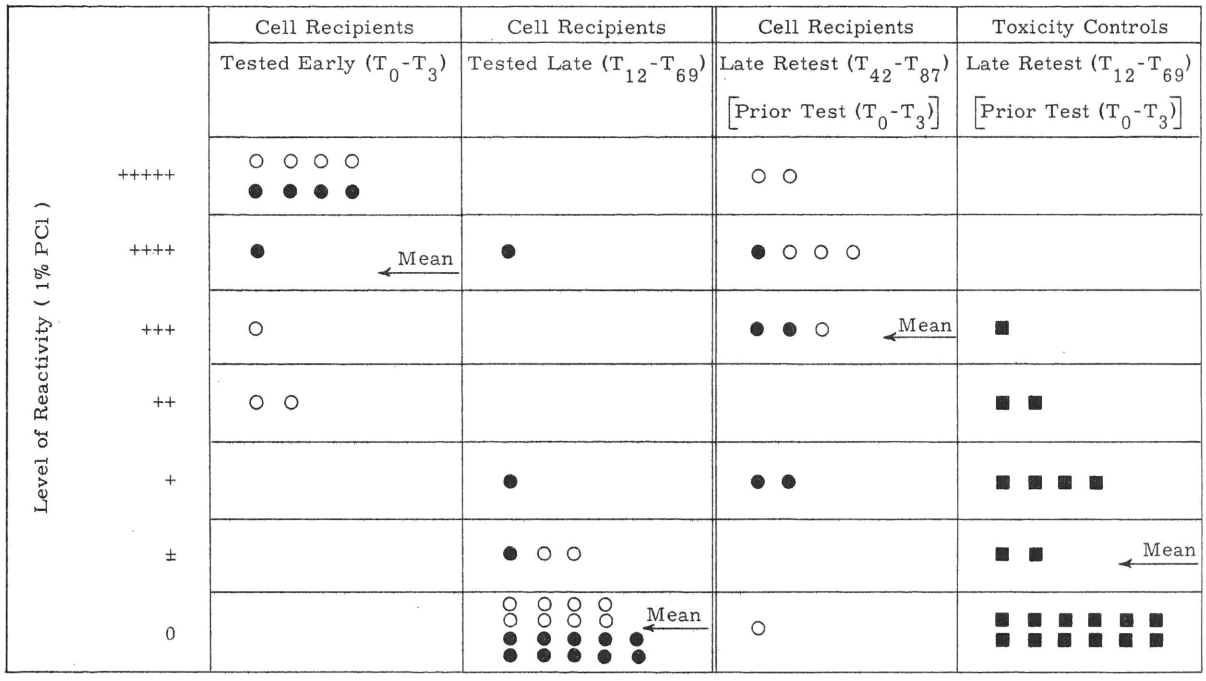
A. Duration of Reactivity in Recipients of Competent Living Cells

It has been reported that human recipients of cells or of cell extracts obtained from tuberculin-sensitive donors have an enduring sensitivity to tuberculin that persists for as long as two years following transfer (Lawrence, 1959). In the former case, the cells actually transferred would not persist for long; in the latter, no cells at all are transferred. Therefore, the interpretation is made there exists a "transfer factor" which can be obtained from competent cells , and can transmit some "immunologic information" from donor cells of one genetic type to host cells of another, and this information is 'self-perpetuating'.

Although experiments on the transfer of tuberculin hypersensitivity in the guinea pig of Metaxas and Metaxas-Buehler (1955) suggested early termination of reactivity of recipients of competent cells, the greater sensitivity of the contact system suggested further experiments might be profitable. Chase (1959) has found that reactivity to contact testing of recipients of competent cells falls into two classes: in one case the sensitivity appears immediately, falls after a few days and is lost after the first week; in the second case, the sensitivity follows this same course except that a second phase of long lasting hypersensitivity appears after the first week, making the pattern of hypersensitivity a bimodal curve. These studies are an attempt to elucidate the factors responsible for these two types of response of recipients of competent cells and to determine more precisely how durable transferred hypersensitivity is in the guinea pig. Competent living cells were transferred to genetically unselected normal recipients which received their first tests at various times between T_0 and T_{87}. In order to exclude the possibility of active sensitization by testing, some animals were not tested at the early times and only the initial tests on these were recorded in Table 11. The first group of recipients of living cells was tested at T_0 to T_3 in order to establish the competence of the transferred cells. The animals reacted quite strongly, irrespective of the cell source and route of injection, the mean reaction score being ' ++++ '. The number of cells transferred varied from 4 to 17 x 10^8 (with the exception of one set of lymph node cells in which 30 x 10^8 cells was transferred into each recipient). When the recipients which had reacted strongly at the T_0 to T_3 tests were retested at later times

Table 11

DURATION OF REACTIVITY IN RECIPIENTS OF COMPETENT LIVING CELLS



○ Cells transferred intravenously

● Cells transferred intraperitoneally

■ Toxicity controls

(T_{42} to T_{69}) they exhibited, again, generally strong reactions, the mean score being ' +++ ', in accord with previous experience. On the other hand, when recipients of the same cells but which were not tested before T_{12} were tested for the first time between T_{12} and T_{87}, it was observed, with only one exception, that the reactions were negative, their hypersensitivity not having persisted. It appears then that the sensitivity of recipients of competent cells lasts only a few days after transfer, possibly only during the live-time of the cells, in agreement with the hypothesis of Metaxas and Metaxas-Buehler that the transferred cells must participate directly in the reaction. The reasons for the sharp drop of sensitivity can be attributed to removal and destruction of white cells by the body in order to maintain homeostasis, and probably to a homograft rejection of the cells, since inbred strains of animals were not used.[1]

It remains, then, to explain the lasting high reactivity of the recipients of living cells which had been tested, only on one occasion, immediately after transfer. It is clear that the reactivity of this group is greater than that of normal second tests on toxicity control animals which also had been tested once previously (on which the mean reaction score was ' $\pm$ '). It will be suggested in the Discussion that this represents a case of increased active sensitization by testing, instigated by the strong inflammatory reaction and cellular infiltration of the first test site.

The results of these experiments are consistent with the notion that the early reactions of recipients of living competent cells are contingent upon some direct action of these cells. There is no evidence for transfer of "immunologic information" which leads to an enduring hypersensitivity or for a factor which is transferred from donor to host cells to perpetuate transferred sensitivity, but apparently by some process of active sensitization it is possible to set up an enduring sensitivity in recipients of competent cells artifactually by a single skin painting.

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[1] It is to be noted that evidence suggests the persistence of sensitivity when cellular transfer is made between genetically similar animals. (Chase, 1963; Bauer and Stone, 1961).

B. Effect of Mitomycin C and Actinomycin D on the Transfer of Contact Hypersensitivity by Living Competent Cells.

It is thus evident that all attempts to transfer hypersensitivity by means of non-viable cells or subcellular material have met with failure in this laboratory. It was hoped that it might be possible to inhibit specifically some defined aspect of the metabolism of the living cells without killing them, in order to study what cellular biochemical functions are needed for their ability to transfer the sensitivity. Recently two chemical compounds have become available for the selective inhibition of the metabolism of mammalian cells, Mitomycin C and Actinomycin D.

Shatkin et al. (1962) have shown that Mitomycin C in relatively high concentrations had the ability to cause depolymerization of nuclear DNA in tissue-cultured L-cells and thus to stop irreversibly RNA and protein synthesis. In lower concentrations, this compound was found to abolish the ability of the cells to divide, but not to affect RNA or protein synthesis. In either case, the cells were able to survive in culture two to three days, by virtue of the fact that certain enzymes necessary for maintenance metabolism have slow turnover times.

The second compound used in these experiments was Actinomycin D, which was found by Reich et al. (1962) to bind to DNA reversibly and consequently to inhibit synthesis of RNA and protein. Actinomycin D appears to act specifically on DNA and, in the case of high concentrations, only DNA-dependent RNA synthesis is abolished.

In our experiments, living cells taken from donors exquisitely sensitive to picryl chloride were incubated in gelatin-Hanks for one to 1.5 hours with and without the compounds (Table 12). With low concentrations of Mitomycin, (10μg/ml) the ability of the cells to transfer was affected only very slightly, if at all in tests made at T_o. All reactions later than T_o were, however, weak or negative as contrasted with the cells incubated without the addition of Mitomycin C. On the other hand, the high concentration of mitomycin (100μg/ml) was found to abolish completely the transfer capacity of the cells, as did Actinomycin (12μg/ml), although no change in the viability of the cells could be detected by trypan blue staining before and after incubation.

It is presumed that at least the RNA synthesis and/or protein synthesis of the transferred cells are critical for the transfer reaction. In addition,

Table 12

THE EFFECTS OF MITOMYCIN C AND ACTINOMYCIN D ON THE TRANSFER CAPACITY OF LIVING CELLS

(Summary Table)

Contact Reactions of Recipients (1% PCl)	Transfer of Cells Incubated Under the Following Conditions‡						
	Gelatin-Hanks		Mitomycin C (10μg/ml) in Gelatin-Hanks		Mitomycin C (100μg/ml) in Gelatin-Hanks		Actinomycin D (12μg/ml) in Gelatin-Hanks
	i.v.	i.p.	i.v.	i.p.	i.v.	i.p.	i.v.
T_o	++++		++++		0		0
T_3		++++		±		0	
Retest T_4	++		0		0		*
Retest T_{12}†				±**	0		
Retest T_{40}†					0	0	

‡ Conditions for a typical experiment will be described: Competent living cells from donors sensitized to picryl chloride by the "Combination Method" were incubated 1.25 hours at 37°C at a cell density of 0.5-1.0 x 10^8 cells/ml with the above compounds, the pH being adjusted with 1.4% unregassed $NaHCO_3$ as required. The cells were sedimented at 230 x g. for five minutes, resuspended (washed once in G-H on occasion) and injected into normal recipients by the route shown, in D/R ratios from 2.6:1 to 4.6:1 of peritoneal exudate, lymph node or spleen white cells. Five experiments are represented. All cell sources reacted alike.

† Animals which reacted strongly to previous test are not included because of the difficulty of determining whether active sensitization had occurred by the previous test.

* Recipients of Actinomycin-treated cells died suddenly three to four days after transfer owing to the toxic effects of the compound, but appeared entirely well at time of test.

** Reaction to Retest T_{12} of toxicity control tested in parallel was ' + ', indicating that the weak reactivity was probably due to active sensitization.

the results of the incubation of cells with the low concentration of Mitomycin C suggest that the transferred cells themselves are directly responsible for the reactions, since under these conditions the cells should have been rendered incapable of dividing. Further experiments will be necessary to accertain that under the conditions of these experiments both RNA and protein synthesis are inhibited by the higher concentration of Mitomycin C and of Actinomycin D and that cells incubated with only 10μg/ml Mitomycin C are rendered incapable of replication.

An earlier effort to block oxidative metabolism may be mentioned here, namely, incubation of competent transfer cells from lymph nodes with 2×10^{-3}M NaCN for 45 minutes at 37° (Appendix Table VI). Such cells proved entirely competent to effect transfer, their reaction to trypan blue was that of viable cells, and their oxygen uptake by manometric technique was precisely the same as that of untreated contact cells. This result is entirely consistent with the later report (Karnovsky, 1962) that leucocytes possess a cyanide-insensitive cytochrome oxidase.

C. The Specificity of Contact Reactions

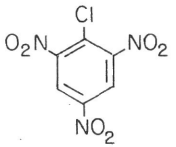
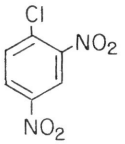
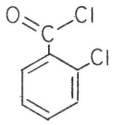
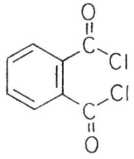
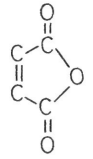
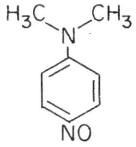
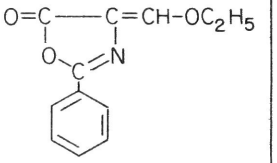
1. Specificity as Observed on Actively Sensitized Animals

Investigation of the specificity of contact reactions on hypersensitive guinea pigs was undertaken initially to find compounds which could be used to detect non-specific reactivity on recipients of cellular material. Consequently, animals actively sensitized to dinitrochlorobenzene and to picryl chloride by several methods were tested with a battery of structurally distinct contact allergens. The compounds were used deliberately, excepting in the case of maleic anhydride, in rather high concentrations which regularly produced trace irritation on normal control animals. It was felt that the sensitivity for non-specific reactivity would be greatest if threshold irritation were present on all animals, in which situation any increases above this background would be indicative of non-specific reactivity.

The results of these specificity tests are presented in Table 13. It was found that, apart from p-nitrosodimethylaniline, no cross reactions were found with the components tested on dinitrochlorobenzene-sensitive animals, sensitized by either the "Combination" or the "Percutaneous" methods (rows 5 and 6). No cross-reaction to o-chlorobenzoyl chloride was found on picryl

Table 13

CROSS-REACTIONS WITH CONTACT ALLERGENS ON ACTIVELY SENSITIZED GUINEA PIGS

	Test Substances †											
	PCl				DnCl		oClBCl	PhthCl	Mal. Anh.	NDMA	Ethoxaz	
												
Sensitization	1%	1/3 %	1/50 %	1/150 %	1/3 %	1/50%	15 %	4 %	5% - 8%	3/4 %	1/5 %	1/15 %
Toxicity Controls	tr	0	0	0	tr	0	±	±	0	±	±	tr
Picryl Chloride												
Intradermal Method	+++±						tr					
Adjuvant Method ††	++++	+++					±					
Combination Method			++++±	+++±			+++	++	±	+±	++++	+±
Dinitrochloro-benzene												
Percutaneous Method	+±				++++		±	±	0	±		
Combination Method			+			+++±	±	+	±	+±		

Legend:
PCl, picryl chloride; DnCl, 2,4-dinitrochlorobenzene; oClBCl, ortho-chlorobenzoyl chloride; PhthCl, phthalyl chloride
Mal. Anh., maleic anhydride; NDMA, para-nitroso-N, N-dimethylaniline; Ethoxaz, 2-phenyl-4-ethoxymethylene-5-oxazolone.

† One capillary drop of vehicle containing the concentration listed.

†† The "Adjuvant Method" refers to the sensitization to contact test with picryl chloride by the injection of picrylated guinea pig erythrocyte stromata in complete adjuvant. The readings to first contact test are given in table. (II C, and Chase, 1954)

chloride sensitive pigs sensitized by the "Intradermal Method" (row 2) or by the injection of the picrylated stromata in adjuvant without subsequent paintings (row 3). On the other hand, an extraordinarily interesting result was obtained in the case of animals rendered exquisitely sensitive to picryl chloride by the combination method (row 4). All of the structurally unrelated compounds produced cross-reactions in some degree, but not one of the order of sensitivity of the specific allergen. (Maleic anhydride was not used here in a concentration adequate to produce threshold toxicity on normal skin.)

These reactions seemingly are attributable, not to a specific cross-reaction between the allergens or haptenic groups involved, but rather to the fact that all of the compounds tested react readily with amino groups and would be expected to couple to and alter guinea pig proteins in a similar fashion. It is postulated that, in addition to a hypersensitivity to the chemical group of the allergen molecule, there is a small component of hypersensitivity to altered guinea pig skin proteins (Chase, 1947). The magnitude of these cross-reactions was not large compared to specific sensitivity to picryl chloride, for it required 15% oClBCl to give reactions equivalent to 1/150% PCl.

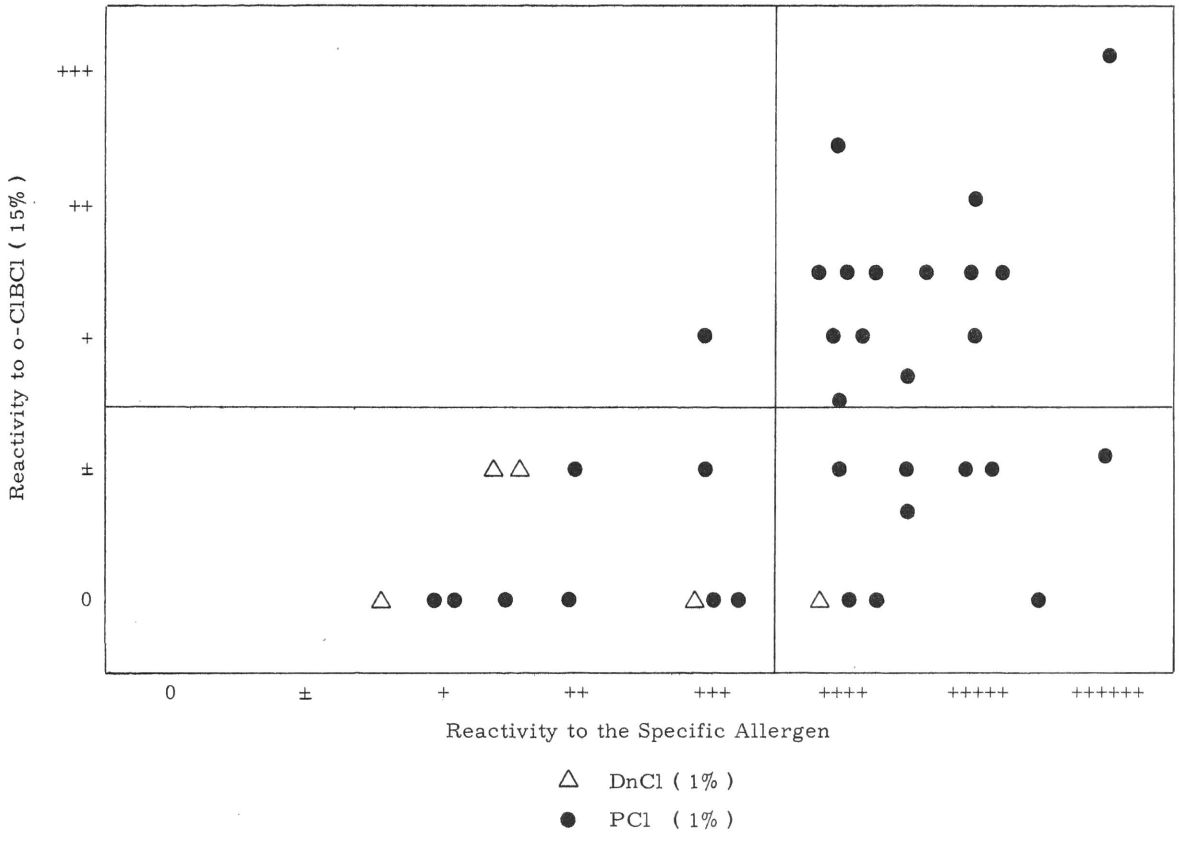
2. Specificity as Seen on Recipients of Competent Living Cells

Previous experience in this laboratory with recipients of living cells had indicated the usefulness of oClBCl in detecting pronounced non-specific hyperreactivity in the earliest stages of "lymph node disease". It was thought that this compound might be used to advantage in measuring the specificity of the contact reactivity following cell transfer in the absence of this disease.

Twenty-nine recipients of competent living cells were tested simultaneously with 15% oClBCl and with the specific allergen involved. As was to be expected on the basis of the reactions above, none of five recipients of cells from dinitrochlorobenzene-sensitive donors showed reactions greater than ' $\pm$ ' to oClBCl, the average reaction to 1% DnCl being about ' $+++$ '. Again, as was to be expected from the results of Table 13, recipients of cells from donors sensitized to picryl chloride by the "Combination Method" gave a proportion of cross reactions (Fig. 2). It is seen that nine out of 32 paired tests (30%), although revealing highly significant sensitivity to the specific allergen,

Figure 2

REACTIVITY OF RECIPIENTS OF LIVING CELLS
TO THE SPECIFIC ALLERGEN AND o-CHLOROBENZOYL CHLORIDE



showed none at all to oClBCl. An additional eight paired tests to picryl chloride showed only marginal reactivity to oClBCl, but there was evident correlation between the two tests in 15 pairs even though the cross reaction was very much weaker than the specific. In 14 such recipients showing ' ++++ ' or greater reactions to 1% PCl, there were 14 reactions out of 23 tests above ' ± ', and the average reactivity of these animals to 15% oClBCl is seen to be ' + ' or ' ++ '. However, of nine reactions of average score ranging from ' ++ ' to ' +++ ' to 1% PCl, only one reaction to oClBCl was as high as ' +'. It is perhaps worth mentioning that in the only three instances in which the ethoxymethyleneoxazolone and o-chlorobenzoyl chloride were tested simultaneously for cross reactivity (ethoxaz used at concentration of threshold toxicity, 0.2% in olive oil) the reactions to these two substances agreed.[1]

The absence of cross-reactivity to oClBCl in the recipients of DnCl competent transfer cells agrees with the data of Table 13, row 6, in which exquisitely sensitive donor animals gave hardly any response to 15% oClBCl. Accordingly, cross-reactivity of picryl chloride sensitive recipients is detectable only on those animals which give the most brilliant reactions to the specific allergen.

3. Justification for Using o-Chlorobenzoyl Chloride to Detect Non-Specific Reactivity in Recipients of Subcellular Materials

It has been shown above that there is no detectable cross reactivity of 15% o-ClBCl in olive oil on guinea pigs sensitized to dinitrochlorobenzene by the "Percutaneous Method" or by the "Combination Method". It is obvious, therefore, that exaggerated reactions to 15% oClBCl on recipients of cells or cell extracts from DnCl-sensitive donors could only indicate non-specific reactivity, since no cross-reactivity is present even in the donors.

In the case of animals sensitized to picryl chloride, no cross-reactions

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[1] Data not reproduced elsewhere in detail may be cited:

Living peritoneal exudate cells, transferred intravenously at D/R ratio of 1.5:1 after incubation for 45 min at 37°C in 10 ml Hanks solution, gave the following responses to test T_o: PCl, +++; oClBCl, +; ethoxaz, +.

Living spleen cells transferred intravenously without preincubation in D/R ratios of (a) 5:1 or (B) 2:1 gave these responses to test T_o:
(a) 0.2% PCl, ++; 15% oClBCl, ±; 0.02% ethoxaz, tr.
(b) 1% PCl, +++++; 15% oClBCl, tr; 0.2% ethoxaz, prac. 0.

to 15% oClBCl were detected in animals sensitized by the "Percutaneous Method" or by the injection of picrylated guinea pig stomata in adjuvant. Cross reactions were indeed detected on animals rendered exquisitely sensitive by the "Combination Method", not only to oClBCl but also to a variety of structurally unrelated allergens. Cross-reactions to oClBCl were found only on recipients of living cells from those donors which gave the most brilliant specific reactions. As stated above cross-reactions on living cell recipients were only rarely to be detected when the level of reactivity of these animals was below the '+++' degree.

On the basis of the data secured both on actively sensitized animals and on recipients of living cells, we can say that the transfer of subcellular materials would not engender any reactivity to oClBCl unless high specific reactivity to PCl were to result from the transfer, since cross-reactivity must parallel specific reactivity (Fig. 2). The degree of reactivity to specific test allergen on such recipients of sonicates and homogenates was never high enough to permit true cross-reactivity to be detected. Accordingly, since it was noted quite often that animals with questionably positive reactions to the specific allergen (Tables 8 and 9) also gave reactions of equal or greater intensity to 15% oClBCl, the latter reactions can only be ascribed to a general non-specific cutaneous irritability or reactivity. Testing with 15% oClBCl in order to detect non-specific reactivity is, therefore, a justifiable procedure, for when positive reactions are found to this compound, any corresponding reactivity to 1% picryl chloride used as the 'specific' allergen becomes highly suspect.

D. Observations on the Nature of the "Complete Antigen" in Contact Hypersensitivity

Interest in the nature of the "complete antigen" involved in contact hypersensitivity is derived from three sources. The results of specificity tests (contact reactions) made on animals that had been rendered exquisitely sensitive to picryl chloride by the "Combination Method" (IV-C) suggested that a component of hypersensitivity was present which could be elicited by all of the allergens used, both specific and non-specific. All of these substances possess reactive groups that are subject to nucleophilic chemical attack by amino groups and the method of contact testing should provide similar chemical alterations in body protein. Secondly, allergen-coupled guinea pig erythrocyte

stromata are so highly effective in sensitizing guinea pigs to give positive contact reactions with the chemical allergen itself that a possible peculiarity of stromata as carrier was suggested. To this was added the knowledge that allergen-coupled simple proteins given with complete adjuvant are relatively ineffective (Chase, 1954) in establishing delayed-type hypersensitivity to the simple chemical hapten. These facts suggested the possibility that allergen-coupled plasma membrane material, as exemplified by picrylated stromata, might play a particular role in stimulating delayed-type hypersensitivity to the simple chemical allergens.

Consequently, a number of substances were coupled with picryl chloride or dinitrofluorobenzene, emulsified into mycobacterial adjuvants, and administered to normal guinea pigs. The effectiveness of the various conjugates in sensitizing the animals was then determined by making contact tests with the respective simple chemical. The results are summarized in Table 14.

Injection of picrylated bovine serum albumin (P-BSA) in the manner described by Benacerraf and Gell (1959) led, in confirmation of their findings, to the development of a low degree of contact hypersensitivity, although our exploration was not wide. In contrast, picrylated or dinitrophenylated guinea pig erythrocyte stromata, when injected with mycobacterial adjuvant, produced a highly significant level of contact hypersensitivity to the allergen. Indeed such results are the reason that it has been used for many years as the routine method for sensitizing donors for cell transfer in this laboratory.

As one model for plasma membranes, myelin, was chosen because much is known about its chemical composition and structure (Finean, 1962) and it has been used often by the biophysicist for studying membrane phenomena. When picrylated myelin was administered similarly in emulsion or simply was suspended in paraffin oil containing mycobacteria, a very strong hypersensitivity to the chemical allergen appeared in two to three weeks. That this effect required mycobacteria is shown in column 7 of Table 14. Myelin not so coupled, injected in complete adjuvant, caused no nonspecific reactivity to picryl chloride (column 8).

It may be asked at this point whether the sensitizing capacity of conjugated stromata and conjugated myelin is due to some special characteristic of plasma membrane materials, or merely represents a non-specific adjuvant-like property of lipid-containing materials. Two experiments were designed

Table 14

SENSITIZATION OF GUINEA PIGS TO CONTACT HYPERSENSITIVITY BY MEANS OF ALLERGEN-COUPLED MATERIALS

	P-BSA	P-GPSt	DNP-GPSt	P-Proteolipid Protein[4]	P-Tbc (H37Ra)	P-Myelin[5]	P-Myelin	Myelin
Amount Injected	2-4μg[1]	750μg	750μg	1mg	1mg	1mg	1mg	1mg
Adjuvant Used	M. butyricum + p.o.[2]	M. tuberculosis + p.o.[3]	M. tuberculosis + p.o.[3]	M. butyricum + p.o.[2]	+ p.o.	M. butyricum + p.o.[2]	+ p.o.	M. butyricum + p.o.[2]
Route	Footpads (0.2-0.4ml)	i.m.[6] (1 ml)	i.m.[6] (1 ml)	i.m.[6] (1 ml)	i.m.[6] (1 ml)	i.m.[6] (1 ml)	1.m.[6] (1 ml)	i.m.[6] (1 ml)
Time of Test	13 days	37 days	21 days	21 days	21 days	16-21 days	16 days	21 days
Reactivity	+ to +++	++++	+++	+++++	0	+++++	0	0

All contact tests made with one capillary drop of 1% of the allergen (PCl or DnCl) in olive oil.

Legend:
P, picrylated; DNP, dinitrophenylated; BSA, bovine serum albumin; GPSt, guinea pig erythrocyte stromata; Tbc, heat killed M. tuberculosis, Strain H37Ra; p.o., medium weight paraffin oil, U.S.P., sterile.

1 - After Benacerraf and Gell **(1959a)**
2 - Difco Freund's Adjuvant (complete) emulsified with an equal volume aqueous phase, final concentration 0.25 mg M. butyricum per ml.
3 - M. tuberculosis, Strain Jamaica No. 22, with paraffin oil and Aquaphor emulsified with aqueous phase (II C)
4 - This injection of picrylated lipid-free "proteolipid protein" provoked experimental allergic encephalomyelitis in three to five weeks. At the time of contact test, animals' behavior was normal.
5 - Picrylated myelin, dissolved directly in paraffin oil with Tbc, but without emulsifier and aqueous phase gave the same result.
6 - Injection of five sites of 0.2ml each into the nuchal muscles on one occasion.

to study this question more closely. Heat-killed tubercle bacilli, a lipid component of which is thought to be responsible for initiating sensitization to tuberculin (Raffel, 1950), were coupled with picryl chloride in the same manner as myelin and were injected into normal guinea pigs as part of a water-in-oil emulsion (Table 15, column 5). The negative results in contact testing, even though the animals became sensitized to tuberculin, suggest that there is, indeed, something special about myelin and erythrocyte stromata which all lipoproteins do not possess. Secondly, a product termed "proteolipid protein", kindly supplied to us, prepared from calf brain myelin by repeated Folch's lipid extraction and lyophilization and possessing no extractable lipid, was conjugated with picryl chloride by the same procedure as myelin. When injected in mycobacterial adjuvant, it appeared to be as effective as picrylated myelin in sensitizing guinea pigs to contact hypersensitivity to picryl chloride (Table 14, column 4).

These results indicate that plasma membrane material, most likely membrane-bound protein, when conjugated with a simple chemical allergen, is effective in establishing high levels of contact hypersensitivity to the allergen, and are consonant with the idea that in contact tests with simple allergen, the active or "complete antigen" seen by the immunologic system may be allergen-coupled plasma membrane protein.

V

GENERAL DISCUSSION

V. DISCUSSION

A. Can Delayed-Type Hypersensitivity be Transferred between Guinea Pigs by Means of Non-Viable Cells or Subcellular Materials?

i.

Many futile attempts had been made in this laboratory to use killed cells to transfer either contact-type sensitivity to chemical allergens or intradermal reactivity to tuberculin from sensitized to normal guinea pigs. Three reports now stand in the literature stating that one or another of these goals is possible. These claims will be discussed below. None of the methods cited in these reports was immediately rewarding in our hands. In order to ensure a systematic approach to the problem, the work reported in this thesis was undertaken.

In contrast to disrupted cells, there is no difficulty in effecting transfer with living cells when one uses proper technique and possesses donor guinea pigs that have attained high levels of hypersensitivity. White cells from lymph nodes, mononuclear-type peritoneal exudates, spleens and peripheral blood all suffice to effect transfer. With the method of sensitizing donors that we term the "Combination Method" (II-C), 0.15 ml. packed cells representing donor: recipient ratios of 1.5:1 to 2:1 per cell source transfer contact-type hypersensitivity well (Plate I, animal C), and ratios of 3:1 to 5:1 transfer a significant degree of tuberculin hypersensitivity. From experience gained with living cell transfers, we conclude that, were it to transfer successfully, non-living material should (a) be capable of giving indubitably positive reactions of clearly greater intensity than any possible primary toxic effects that may be seen occasionally in applying similar tests to any normal guinea pig;[1] (b) induce hypersensitivities that show a specific relationship with

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[1] In this laboratory, the records present adequate verbal descriptions of all reactions, even feeble ones; typical readings of trace reactions, for example, may be cited: "few scattered small pp spots on vfp background"; "two or three fp coarse spots, vfp much mottled" "very many scattered p and pp/p dots, especially on dorsal half of site". The original readings are not recorded merely as an increase over reactions of the controls. For purposes of presentation here, the actual descriptions of reactions have had to be transposed into symbols. The question arises as to why so many workers report their controls to be ' 0 ' or ' negative '. We wonder about ' 0 ' being reported (seen?) on guinea pigs tested with 1:4 O.T., for example. Can it be that others tend to overlook reactions that at a glance are not meaningful and therefore are simply recorded as ' 0 ' ?

the inciting allergen, similar to the results of tests for specificity on actively sensitized guinea pigs used as donors; and (c) be encountered repeatedly when the same technique is employed. Further the use of chemical depilatory should not be mandatory to produce positive reactions since no depilation is required when living cells are transferred. Since a dose-response curve is seen with the transfer of living cells, we would anticipate encountering this also in effective transfers of non-living materials. One might expect the efficiency of transfer following cellular disruption to be lower than in the case of living cells. The use of larger amounts of material in the case of disrupted cells would, at the same time, be expected to intensify the level of reactivity should it be transferrable. (To allow for expected inefficiency of transfer by subcellular materials, we increased donor:recipient ratios from twice to twenty-fold over the amounts of living cells effective for transfer, yet experiments were included using low D/R ratios with subcellular materials (Table 5).)

From the results of our investigations reported above, it is evident that not one recipient of lethally damaged cells satisfied the several criteria described here, in transfers involving 128 recipients in about 33 large experiments under varying experimental designs and with donors sensitized in either of three ways. Cells for these transfers came from 279 tested and demonstrably highly sensitized guinea pigs, peritoneal exudates being taken from 235 animals in 14 experiments, lymph nodes from 212 animals in 15 experiments, spleens from 254 animals in 17 experiments.

More than 108 separately treated portions of living cells were used, with cells of each source handled individually unless specifically stated. Fifty-three transfers of living cells made in low donor: recipient ratios served to validate 93 of the 108 portions described here. Of the 53 transfers, 49 conveyed brilliant sensitivity and only three (ratios of 2:1, 2:1 and 3:1) failed to issue positively, although in all instances other cell sources from the same donors gave entirely acceptable transfers.

The 15 portions of cells that were not controlled by concomitant living cell transfers (PC1 463, 464) were drawn under equivalent conditions from donors shown to be individually as highly sensitive as those animals whose cells were specifically validated.

Transfers of sonicated material were performed on 88 recipients, materials obtained by homogenization on 21 recipients, and repeatedly frozen and thawed cells on 19 recipients (Tables 6 and 7). Attempts were made to effect sensitization by transferring subcellular material intraperitoneally, intravenously, and intradermally. Materials which

might offer protection for liberated materials such as yeast RNA, cysteine and serum were added to the sonication media. Sonically disrupted cells were injected into certain recipient guinea pigs having an established peritoneal exudate. Sonicated material was also preincubated with mononuclear-type peritoneal exudates of normal guinea pigs in hopes of facilitating transfer (Appendix Table VI). In one experiment, sonically disrupted cells from contact hypersensitive animals were emulsified in Freund's complete adjuvant (PC1 467-A, Recipient 15) without evidence of induction of dermal hypersensitivity or antibody production (Appendix Table VI).

Among these 128 recipients of disrupted cellular materials (Tables 6 and 7)[1], 27 individuals showed reactions scored ' ± ' or greater at one or more tests, and these are analyzed in Tables 3, 8, and 9. There can be excluded at once 9 individuals, two because their pretreatment as intended recipients provoked general irritability (Table 8, first two recipients), four because sonication was so brief that evidently living cells were present (Table 3), and three because they, like their toxicity control counterparts, had become sensitized as a result of their earlier contact tests. Of the remaining 18 individuals, the minor reactivity of 17[2] can be explained satisfactorily on one or more of the following bases (Tables 8 and 9):

(a) corresponding normal animals ('toxicity controls') showed unduly high minor reactions in the same experiments;

(b) unduly high reactivity to contact tests made with o-chlorobenzoyl chloride as a chemically distinct test allergen;

(c) other recipients given the same material in equal or greater amounts were clearly unreactive.

There was utter randomness in the distribution of the 'reactor' individuals and unlikeness in the reactions occurring on adjacent days. The distribution in the responses of the 54 toxicity controls to contact testing was tabulated and is shown in curve A, Figure 3. Each contact test with

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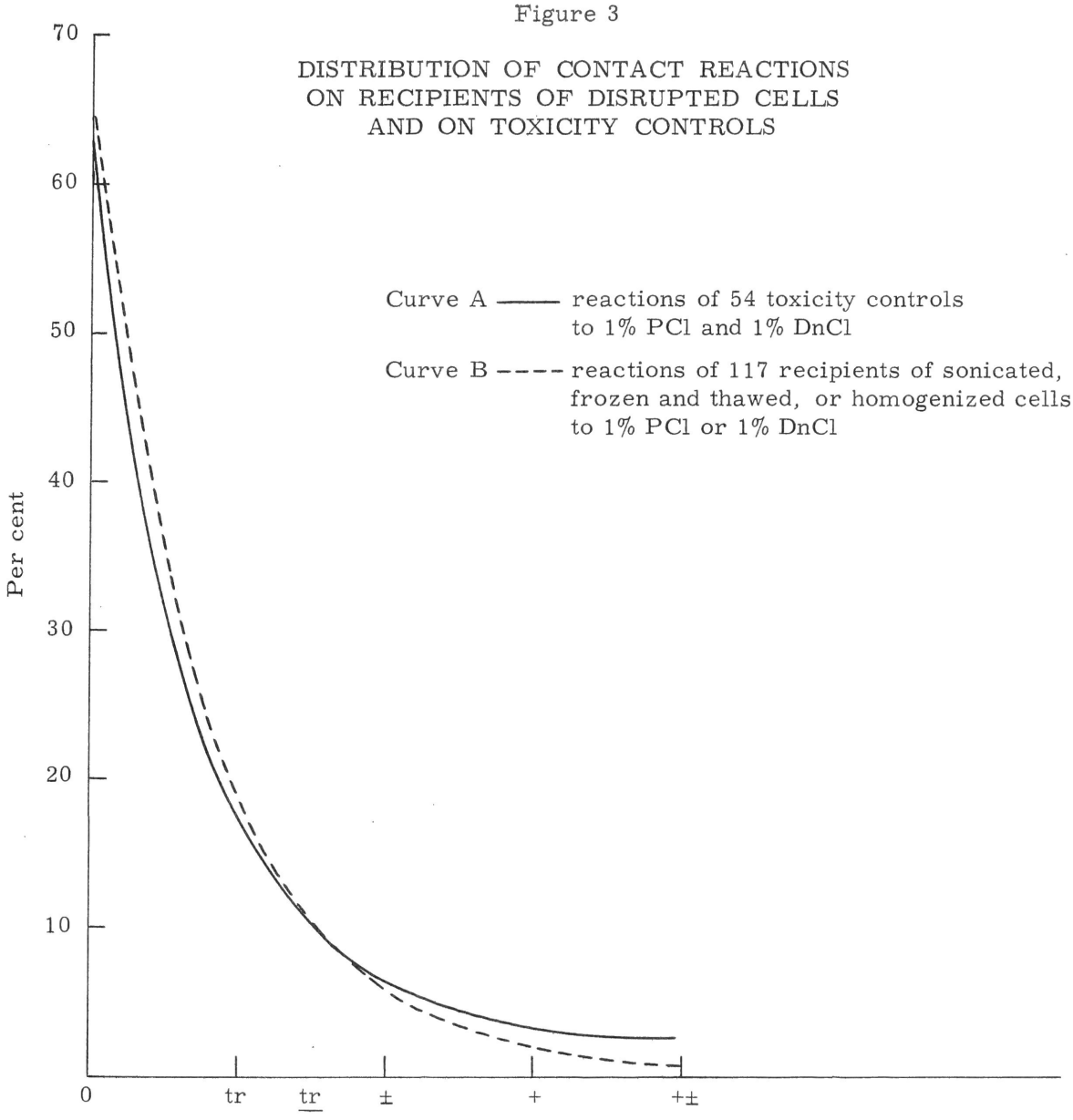
[1] Four of the 128 recipients are omitted from these tables since only tuberculin testing was performed (III-B and Appendix Table VII-b).

[2] The actual scoring of the highest "reactions" seen on these 17 individuals was: tr, becoming ± (3 instances); ± (8instances); ±; tr or ± becoming + (two instances), ± becoming ++; ++; ++ becoming ++. On none of these animals was this 'highest reading' present at more than one single test. Of these animals, nine were among animals receiving sonicated materials, four among animals receiving frozen and thawed cells, and four among animals receiving homogenates.

Figure 3

DISTRIBUTION OF CONTACT REACTIONS
ON RECIPIENTS OF DISRUPTED CELLS
AND ON TOXICITY CONTROLS



picryl chloride or dinitrochlorobenzene made on non-depilated sites of the animals was recorded, never more than three tests on one animal being accepted, and then only if the sites were made on successive days; 129 such tests are represented. It is not to be assumed that the response curve reflects solely the irritability of the Rockefeller Institute albino guinea pig colony, since irritative reactions result from a variety of causes (III, p. 38) and they tended to cluster in certain experiments, e.g., Appendix Table X. Yet the observations, spread over many months, offer a clear picture of the range of responses that are encountered.

When a similar formulation was made of the test readings of 117 animals that had received non-living materials prepared from competent cells,[1] Curve B (Figure 3) resulted, based on 250 contact tests. This curve is indistinguishable from Curve A, partly fortuitously, but certainly a significant displacement would be expected if actual transfer was being encountered even if low-grade. This result indicates that reactions of recipients given extracts of competent cells distributed themselves statistically in the same way as the reactions given to the same test materials by normal, untreated guinea pigs.

We conclude, consequently, that there remains only one individual among the many recipient animals that requires evaluation as a possible instance of positive transfer which appeared in an experiment performed by Goldstein, Chase and Bloom. (PC1 462, Recipient 2, Table 8) There exists no question as to the quality of the reaction observed. It will be well to recount the procedure for this transfer in detail.

> Peritoneal exudate cells (0.55ml packed volume) were suspended in 4.07ml 10% guinea pig serum in Hanks solution. There was added 0.81ml of 0.7M cysteine in physiological saline (liberated from cysteine-HCl by neutralizing to pH 7.8), 0.56ml 4% yeast RNA in physiological saline (pH 7.7), and 0.16ml guinea pig serum. The final serum concentration was 10% and the final cell suspension about 9.7% (v/v). The suspension read pH 6.0 and was brought to pH 7.65 with use of NaOH. After three minutes of sonication by the indirect method in a polyethylene tube, there remained very many intact cells of which only about one-third showed nuclear staining with trypan blue. After a second period of sonication for an additional 3.5 minutes, almost all of the cells appeared rather abnormal, but not all were disrupted. A third period

[1] The 9 individuals excluded earlier and Recipient 2 of PC1 462(to be discussed separately) are not included here.

of sonication followed, for 2.5 minutes, after which the suspension was rather clarified, but some cells were still morphologically intact although showing nuclear staining with trypan blue. The entire suspension (effective donor:recipient ration of 5.8:1) was injected by the intraperitoneal route into an albino male guinea pig (408g). For unknown reasons, this animal was not well for a few hours and remained partly prostrate. Thereafter, it behaved normally. The first contact test (T_1) was made 33 hours following the transfer.

Tested in parallel were three other recipients, one having received by the intraperitoneal route another sonicate of an identical volume of the same cell pool (0.55ml cells in 4.07ml 10% guinea pig serum in Hanks solution, plus 1.58ml normal guinea pig serum to 35% serum, requiring only five minutes sonication to disrupt). The other two recipients had received lymph node cells from the same donors, frozen and thawed in different ways and described adequately in Appendix Table III-B. A recipient of living cells from the same peritoneal exudate cell pool at only a 1.5:1 D/R ratio was also tested in parallel. The living cells had been held for four hours at 4°C in 1.5ml of 7% normal guinea pigs serum Hanks and were injected intravenously.

No toxicity controls were available owing to ill-advised changes in stock breeding in the Rockefeller Institute albino guinea pig colony which required a year to rectify.

As expected, the recipient of living cells validated the cell source, showing a ' + ' reaction at nine hours and a ' ++++ ' at fifteen hours. Recipient 2 at fifteen hours had a slight reaction to test T_o, patchy, but judged "probably positive". The T_o reaction site was ' ++++ ' at 36 hours, and contact test T_2 was scored ' +++ ' at 20 hours, ascending to ' ++++ ' at 46 hours. Tuberculin test T_2 with 50μg of PPD was, at most, equivocal. Recipient 3, which received the same cellular material sonicated in 35% serum Hanks remained inert to all tests, as did the recipients of frozen and thawed lymph node cells.

Questions that remain are (1) whether the response of Recipient 2 was sensitization effected by means of living cells, or (2) whether by genetic chance, any process resembling "lymph node disease" (although improbable under these circumstances) might have occurred such as to give non-specific reactivity.

At the time, this response was accepted, although running counter to prior experience, as positive transfer, and it was supposed that it could be repeated in future trials. The difference in response between Recipient 2 and Recipient 3 was then attributed to the presence of RNA and cysteine in the medium. But it turned out that repetition was not possible, in seven further attempts in which the same medium was used, and in eight other attempts in which the same medium lacking RNA was employed (Table 6).

The possibility that living cells had been transferred seems not implausible. First, successful transfers have been achieved with surprisingly small volumes of living cells, perhaps one-fifth to one-fifteenth of the amount

sonicated here. Secondly, at about the time of this experiment (December 1959 and continuing through May 1960) the behavior of the 10kc, 200 W Raytheon oscillator was erratic. It will be noted that "disruption time" for Recipient 2 was 3.0 minutes plus 3.5 minutes plus 2.5 minutes and that morphologically intact cells were still present although we felt that all nuclei were being stained with trypan blue. In contrast, the equivalent batch of cells sonicated separately in 35% serum Hanks was disrupted completely in five minutes, no intact cells being observed. (In the following two experiments sonication times of 10 minutes and 8 minutes were required; the machine was then serviced, and sonication times for equivalent disruption dropped to between 3 and 4 minutes.)

Mention has been made that very weak positive reactions to contact testing tend first to appear as a spotty distribution of faintly pinkish patches, probably over the larger vessels in the skin. Recipient 2 showed this type of pattern, and it would be expected that any very weak reaction would start in this fashion. This was not the case, however, as seen in irritative reactions that have been recorded, for example in the lower part of the curves in Figure 3. Even allowing that Recipient 2 might have represented a successful transfer with non-living materials effected in unknown way and therefore not reproducible, the type of reaction seen was so different from that of the other recipients previously discussed that these others could not be accepted as representing positive transfers.

ii.

In the experiments described, we saw no clue suggesting that a way to effect transfer with non-living materials had been found. It may be well next to recount those experiments that were carried out to repeat in detail the work reported by Jeter et al. (1954) claiming positive results with the use of sonically disrupted peritoneal exudate cells from dinitrochlorobenzene-sensitive donors. Indeed, this report had been the stimulus for resuming attempts to transfer contact hypersensitivity by means of disrupted cells.

The main points in the work by Jeter et al. are as follows: (a) sensitization of donors to dinitrochlorobenzene by the "Percutaneous Method" (II-C); (b) securing peritoneal exudate cells on the twelfth to sixteenth day; (c) sonication in gelatin-containing Locke's solution in a Raytheon 9kc, 50W Oscillator by the "direct method" (30°C);

(d) injection of sonicated material from four to eight donors, either entire or centrifugal fractions, by the intraperitoneal route; (e) chemical depilation of skin sites 24 to four hours prior to testing at T_2; and (f) reading the contact sites one and two days later. The scale used for recording the reactions was higher than the one that we employ (Plate I), as judged from Dr. Jeter's Kodachrome pictures. It is also to be noted in the latter's experiments that the active material was associated with the supernatant fluids of sonicated materials and not with the centrifugal sediments. Sonicated material appeared not to transfer tuberculin hypersensitivity.

It should be noted that in the report of Jeter et al., reactions of recipients of disrupted cells were judged to be quite comparable to those of recipients of corresponding amounts of living cells, that erythema was the sole criterion reported for reactivity, that no mention was made of control tests on normal animals, that specificity testing was not performed, and that 'positive results' were encountered only irregularly[1] both with living cells and sonicated material.

In our series of percutaneous sensitizations to dinitrochlorobenzene, we confirmed the reactivity of the intended donors by skin tests and secured cells five days later (on day 22). High transfer capacity of living cells was demonstrated. Thirteen normal recipients were used in attempts to transfer, seven receiving sonicates of peritoneal exudate cells, four of lymph node cells, and two of spleen cells (Table 2: DnCl 280, 283-A). Our suspension fluid for sonicating was gelatin-Hanks; Jeter's was gelatin-Locke's. Cellular breakdown during sonication was carefully followed. Entire sonicates were transferred five times and sonication supernatants and sediments four times each. Sonication was performed comparatively in DnCl 283-A (Appendix Table I-b) by disrupting part of the pool of lymph node cells for 10 minutes in the 9kc, 50W Raytheon by the "direct method" (material equivalent to five to nine donors per recipient) and part in the 10kc, 200W Raytheon by the "indirect method" for times ranging from one to six minutes, with donor: recipient ratios of 6:1 to 9:1 being transferred. Of 35 contact tests made on these 13 recipients of sonicated with the specific allergen on clipped skin areas, only two recipients require comment. Recipient 6, DnCl 280, received material after 2.5 minutes of "indirect" sonication that contained

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[1] Personal communication, Dr. M. W. Chase

intact cells (Table 3). Recipient 6 of DnCl 283-A reacted ' ++ ' vs toxicity controls of ' tr, ±, ± ', with non-specific reactivity towards o-chlorobenzoyl chloride of ' ± ' vs toxicity controls of ' tr, ± '. The slightly greater reactivity of this animal is to be compared with the four other recipients (Nos. 3,4,5, and 7) receiving material from the same pool.

Later, we turned from animals that had been sensitized percutaneously to donors that were much more highly sensitive to dinitrochloribenzene, prepared by the "Combination Method" and reacting well to 0.02% (average '++++ ') down to 0.01% or less in olive oil. Living peritoneal exudate cells and spleen cells in DnCl 283-B transferred well, and spleen cells in DnCl 284-A; other cell sources, probably for technical reasons, failed to transfer well. Confining our attention to the instances in which living cells were highly competent, cells were sonicated directly in the 9kc, 50W Raytheon for 10 to 15 minutes as used by Jeter et al.: both the supernatant and the sediment from 18 x 10^8 peritoneal exudate cells were inactive (2 x 10^8 living cells were active); also, entire sonicate of 27 x 10^8 white cells from spleens was inactive (6 x 10^8 living cells produced good transfer); similarly, 43 x 10^8 white cells of the spleen, sonicated, yielded inert supernatant and cell sediment while 9.5 x 10^8 living cells gave brilliant transfer (Appendix Table II).

The remaining experiments on sonication were conducted on cells taken from donors rendered exquisitely sensitive to contract with picryl chloride (0.0006% or less) by the "Combination Method". A summary of these experiments was given previously in the Discussion; here, we need draw attention only to the use of sonication temperatures in the range used by Jeter et al., as contrasted to the range of 0° to 6°C that we chose for most experiments (II-H) in order to minimize autolytic effects following cellular disruption.

In PCl 477, sonication conducted at 30°C failed to transfer hypersensitivity (total sonicate of 25 x 10^8 splenic white cells and sonication supernatant and sediment separately of 37 x 10^8 peritoneal exudate cells. Other temperatures (17°C, 40°C) in PCl 465 were no more successful.

The experiments being unrewarding, a study was next undertaken to determine whether the 'positive transfer reactions' of this group could be an artifactual result of the use of chemical depilatory. As was mentioned earlier (III-C) on the basis of previous experience in this laboratory, treatment of

skin sites with chemical depilatories produce definite irritability of the skin, and hence it has been avoided as a most hazardous procedure. Indeed in the light of all of our experiences it seemed strange that the authors cited had never encountered any but equivalent reactions after injecting either living cells or sonicates of equal aliquots.

A comparative study was made of reactions to contact testing on pre-depilated skin and on skin that only had been clipped free of hair (cf. II-D). On recipients of competent living cells, we found greater reactivity in simultaneous testing on pre-depilated skin than on normal clipped skin (16/18 instances). Further investigation revealed that this was a summation of specific reactivity and greatly heightened non-specific reactivity. In animals lacking all specific hypersensitivity, normal 'toxicity control' animals for example, the removal of hair stubble by a thioglycollate-containing depilatory as used by the Jeter group prior to testing increased the frequency of irritative reactions (scored ' $\pm$ ' or greater) by about 50-60%, and was observed both with PCl or DnCl used as the 'specific allergen' and with oClBCl as a non-specific irritant (Table 10). These results were indeed illuminating and pointed clearly to the hazard involved in the use of chemical depilatories. (III-C). When tests were made similarly on recipients of sonicated materials, there was no difference from normal control animals in the frequency or intensity of these irritative reactions. Whereas only one out of five or six tests on skin carefully cleared of hair with an electric clipper showed any irritative reactions to 1% PCl or 1% DnCl, 60-70% of tests made on pre-depilated skin gave reactions scored as ' $\pm$ ' or greater; of the latter, about 50% might have been adjudged as positive had they not occurred on normal animals to the same degree and with the same frequency.

Inspection of the data of DnCl 283-A (Appendix Table I-b) shows that, had only test T_3 been applied to pre-depilated skin, some weak degree of transfer would have been inferred if toxicity controls had not been included. But a parallel T_3 test that was made simultaneously on skin freed of hair by electric clipping alone argues quite to the contrary. Recipients 6 and 7 are discussed in Table 8 since they showed both slightly higher reactions than the others and reacted abnormally highly to the non-specific allergen oClBCl. (At the same time it will be noted that on pre-depilated sites there would have been as much reason to attribute 'reactivity' to sediment as to sonication supernatant, or for that matter to normal controls.)

It can be seen that the report of Jeter, Tremaine and Seebohm (1954) was not confirmed in these experiments. In addition, variations on their method such as using more sensitive donors, different media for sonication, different sonic oscillators, other cell sources and routes of injection produced no indication that contact hypersensitivity could be transferred with sonically disrupted cells.

iii.

The second report standing in the literature regarding transfer between guinea pigs with use of sonicated cellular material is the very curious paper by Cummings, Patnode and Hudgins (1956), which appears to have no substantive weight. Living peritoneal exudate cells were taken from donors 30 days after intramuscular injection of 0.3mg killed M. tuberculosis H37Rv and shortly after skin testing. Injected living, in donor:recipient ratio of 10:1, there was, in our judgment, no evidence of any transfer. The purported 'reactions' at 24 hours to test T_2 employing 0.1ml 1:4 O.T. were 2 x 2 mm in diameter and were read as 6 x 7 mm on the next day; apparently no toxicity controls were injected in parallel. Exactly the same size 'reactions' were observed in guinea pigs that had received supernatant fluid from an equivalent amount of cells sonicated for 15 minutes at 30°C. In a second experiment employing donor:recipient ratios of abour 5:1, the single T_2 test with 5μg PPD on the recipient of living peritoneal exudate cells showed at 24 hours a 7 x 8 mm site which might possibly have been a positive reaction. Prior and later tests of their one recipient of living cells we believe to have been negative (according to the experience of this laboratory with 1:4 deglycerinated Old Tuberculin). Corresponding sonicate supernatants can not be accepted as having transferred hypersensitivity as the authors alleged, nor can their homogenate or sonic extract of spleen cells. It is felt that one must be particularly critical of toxicity reactions in experiments of this type. Living cells transferred by the intraperitoneal route produce ascending induration in T_2 test sites over a two-day period, usually without ascending diameter. The absence of any description of induration is a serious omission of the report by Cummings *et al*.

Addendum

Since completion of the work presented herein, the report by Oprescu (1962) has been brought to our attention. Quite unlike our finding that no transfer was possible by injecting intact normal cells from five normal donors after preincubation with sonically disrupted cells from five sensitized donors (described on p. 47 and presented in Appendix Table 6, Recipient 2 and pertinent toxicity controls), Oprescu claimed to have transferred tuberculin hypersensitivity by this principle.

Guinea pigs were sensitized over a 4-week period with 5 mg. dead tubercle bacilli and 30 mg. living BCG. Cells from 4 tuberculin-sensitive animals were taken 5 days after injecting Eagle's solution with 1% glycogen (20 ml) and paraffin oil (1 ml). These cells were divided into 3 lots. One-third was sonicated for ten minutes and the supernatant was incubated at 37°C for 1 hour with leukocytes of 4 normal guinea pigs. Another one-third of the competent cells was handled similarly, after disruption by fifteen successive freezings and thawings. The final one-third was treated for 5 minutes with sodium desoxycholate (1%), and then dialyzed.

In each instance, the cells were washed and injected intraperitoneally into 5 'normal' but tuberculin-pretested recipients, each then receiving material corresponding to not more than one-fifteenth of the cell yield from the primary donors. After a certain time (not stated), all animals were tested with 0.1 ml 1:20 Old Tuberculin, in comparison with animals that had received normal cells after incubation with some disruption product from normal cells. Reactions were seen in all animals excepting the controls, the smallest (3 x 3 to 5 x 6) occurring with desoxycholate-treated cellular material, the others running from 7 x 7 to 9 x 14 but being curiously short-lived: the 48-hour reactions ran from 2 x 2 to 5 x 4.

We would make the following comments about this work, which seems negated by our own experiments. In particular, we are reluctant to pretest normal guinea pigs with Old Tuberculin since the latter can initiate sensitization.

1. Had the cells from the sensitized donors been transferred intact at donor:recipient ratios of 0.4:1, the resulting reactivity should not have been sufficiently characteristic to allow sure interpretation by gross inspection.

2. The reactions observed by Oprescu were of equal size both at 8 and 24 hours, dwindling markedly by 48 hours. But in our own experiments with living competent cells injected intraperitoneally, the reactions develop over a period of perhaps 30 hours and the size and induration at 48 hours are still of considerable magnitude.

3. From our own experience with injection of Old Tuberculin, we would have expected trace reactions in normal controls.

4. Reasonably large numbers of competent cells were used in our experiment of Appendix Table VI, which was negative in outcome. Incidentally, it will be observed that 50 mgm. PPD caused slight irritative reactions of equal degrees in both normal controls and in recipients of living cells preincubated with and injected with sonicated cells.

5. No transfer was secured by injecting sonicated cells into a recipient that was preinjected with sodium caseinate to provoke a normal cellular exudate (Appendix Table VI, recipient 1-Sp.).

iv.

When the present work was well advanced, there appeared a report by Turk (1961) stating, to our surprise, that the cells of donors sensitized to picryl chloride by the "Combination Method" could be disrupted at will and still transfer picryl hypersensitivity by use of frozen and thawed cells, by sonicates, and by cell sap and microsomal fractions of cell homogenates. Our previous experience, negative as seen above, covered all but cell homogenates.

Accordingly, the experiment on homogenization was repeated in detail by transferring cell sap and microsomal fractions, again to no avail (PCl 491-A; Appendix Table IX).

Dr. Turk was kind enough to visit this laboratory and to conduct with us two other large transfer experiments using his precise methods.[1] The results supported our conclusion that specific hypersensitivity was not transferred by this method (15% oClBCl testing disclosed that three recipients which had exhibited trace reactivity when tested with picryl chloride were non-specifically reactive (Table 9; Appendix Table X). In all, of 11 recipients receiving cell sap fractions intravenously and 10 animals receiving microsomes by the intraperitoneal route, there was no instance in which hypersensitivity was seen to be transferred with homogenates, tests being made at T_o and at T_2; 17 further transfers of sonicated cells injected intravenously, rather than intraperitoneally, were negative likewise.

Subsequently, Dr. Turk used another compound for detecting non-specific reactivity, namely, 2-phenyl-4-ethoxymethylene-5-oxazolone, which has led him to withdraw his previous claim because the slight reactivity which he interpreted previously as transfer he now recognizes as being non-specific (Turk and Asherson, 1962).

> In the original work of Dr. Turk, contact sites were depilated at 24 hours with a barium sulfide paste before reading, and the highest score of the 24 or 48 hour readings was published. It turned out that the 48 hour reading was almost always greater than that made at

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[1] In PCl 492-A, the suspending medium that Dr. Turk had actually used was employed (1 part 5 x Askonas stock, 4 parts Hanks solution). In PCl 492-B, the actual Askonas low-electrolyte, sucrose suspending medium was chosen.

24 hours and this suggested to us that heightened irritative reactions of the skin due to the chemical depilatory might be involved. In experiments designed to clarify this point, tests were made on clipped skin, and at 24 hours, just after making the readings, one-half of the area of each site was carefully depilated (III-C) with a thioglycollate depilatory. When these sites were read 24 hours later, again it was found that the frequency of irritative responses was about 60% greater on the depilated half than on the undepilated half of these same test sites, both on recipients of cell fractions of homogenates of competent cells and on normal animals serving as toxicity controls (Table 10, Part C).

We are therefore led once again to the conclusion that chemical depilation is an entirely unsafe procedure to employ routinely in studies on contact hypersensitivity, often leading to a non-specific cutaneous irritability. Depilated sites after the passage of some hours should never be considered to show meaningful reactions. While depilation can increase or magnify weak specific reactions, as after transfer of small amounts of living cells, it will magnify any non-specific irritation, from the chemical reagent o-chlorobenzoyl chloride to occasional scratches made by animals on depilated sites.

V.

By way of summary of the work up to this point, it may be said that of 128 recipients of sonicated, frozen and thawed, or homogenized cells or fractions obtained from competent peritoneal exudate, spleen, or lymph node cells, there was only one instance in which possible transfer of hypersensitivity might have been accomplished. Not one of these recipients fulfilled the criteria established for proven transfer of hypersensitivity, i.e. that the reactivity of the recipient be above the level of toxicity of the allergen on normal guinea pigs and that the reactions be shown to be specific. It was shown that testing should not be subjected to the hazardous effects of chemical depilation of the skin sites.

Obviously a successful procedure for transfer with non-living material should be repeatable in any laboratory. While we cannot presume to deny the possibility that delayed-type hypersensitivity some day may be transferred among guinea pigs by subcellular materials, we can insist that no reproducible method for doing so has yet been found.

The negative findings of these many attempts to transfer delayed-type hypersensitivity in guinea pigs stands in sharp contrast to the reported positive results in man. In man, simple extracts of frozen and thawed cells are said to produce long-lasting hypersensitivity. We would like to see a

well-designed experiment in man with use of cellular extracts in which (a) pre-testing with the allergenic material to validate the negative or anergic state of the recipient could be avoided; (b) some human recipients would be tested for the first time within two days while others would not receive their first test for various times, say one and two months; and (c) an allergic system should be used in which one could be sure that the normal recipients had not been stimulated with the chosen allergen. To accomplish this design, multiple volunteer subjects as recipients and many donors would be needed; some subjects would have to be tested in parallel with the recipients as "normal controls". Yet such a laborious effort is the only way in which it has been possible to explore and deny the transfer of contact hypersensitivity between guinea pigs with subcellular materials.

Another approach to the problem was modelled after an experiment conducted with human beings (Lawrence and Pappenheimer, 1957). It was reported that cells capable of transferring tuberculin hypersensitivity will, upon exposure to PPD tuberculin in vitro, lose their capacity to transfer tuberculin hypersensitivity and free into the medium "transfer factor", the cells remaining viable. The latter, even though present in PPD-containing medium, is said to sensitize human recipients to tuberculin. Indeed, when human cells having two competencies (transfer of delayed hypersensitivity to diphtheria toxoid and to tuberculin) were exposed to tuberculin, the only loss from the cells was that of their tuberculin competency (Lawrence, 1959). Unfortunately, the reverse experiment was not tried.

The same plan was carried out with three cell sources of tuberculin sensitive guinea pigs, mononuclear peritoneal exudate cells, lymph node cells and splenic cells. (Appendix Table XI). The findings were entirely unlike the reported human results.

> Exposure of peritoneal exudate cells to PPD during a two hour incubation period at 37°C in enriched culture medium (9 x 10^8 cells in 12ml; PPD, 3.3μg/ml) did not reduce in discernible degree the capacity of these cells, washed free of tuberculin, to transfer tuberculin hypersensitivity, as compared with another aliquot of the peritoneal exudate cells incubated without tuberculin (III-D). Neither did three hour incubation of 9 x 10^8 cells in 1:200 dialyzed Old Tuberculin alter the capacity of the living cells to transfer effectively. Likewise, incubation of lymph node cells with dialyzed Old Tuberculin for two hours under the same conditions removed none of the capacity for transfer (10.3 x 10^8 cells, in 12 ml containing 1:200 dialyzed Old Tuberculin).

Still another portion of peritoneal exudate cells held within dialysis tubing (with three volumes of reinforced Eagle's medium outside during 3 hours of incubation at 37°C) did not allow "transfer factor" (tuberculin free) to pass through the sac.

The above experiment, done without the addition of fresh serum, gave exactly the same result as another experiment (PC1 467, Appendix Table VI) in which 10% fresh serum was used. Splenic cells from 6.5 donors sensitized by the "Combination Method" both to picryl chloride and to tuberculin were incubated from 1.75 hours at 37°C with 25μg PPD in 10 ml 50% guinea pig serum-Hanks (1.5 the serum was freshly drawn). The cells retained their ability to transfer both picryl chloride and tuberculin hypersensitivity, whereas the culture supernatant was negative to both tests.

If the contact of tuberculin with tuberculin-competent cells actually 'removes' the tuberculin transfer capacity with human cells, it would be expected to represent a general principle. As is seen above, competent guinea pig cells treated with PPD or Old Tuberculin retain fully their capacity to transfer, hence a general principle does not appear to be involved. Perhaps the human experiments should be repeated.

B. What is the Duration of Acquired Hypersensitivity in Recipients after Transfer of Competent Living Cells?

As mentioned earlier, these studies were stimulated by three sets of experimental findings. In transfers of living cells from human donors highly sensitive to tuberculin, maximal reactivity of recipients was found to occur after various times (2 days to 4 weeks) and in most cases was found to persist for as long as a year or more. Since the transfer is said to be accomplished without the use of whole cells and the persistence of skin reactivity is similarly durable, Pappenheimer and Lawrence postulated that a biochemical moiety isolable from competent cells termed "transfer factor" was responsible for producing reactivity in previously tuberculin negative human recipients. The duration of this transferred sensitivity led Lawrence to postulate (1959) in the case of tuberculin hypersensitivity that

> "transfer factor represents an expression of the imprint that the degradation of the specific bacterium imposed upon the biosynthetic functions of the blood-forming stem cells or reticuloendothelial cells of the sensitive donor at some time in the past. The nonreproductive and transient life span of the peripheral blood leucocyte would in turn suggest that such altered stem cells continually confer on newly formed leucocytes released to the peripheral blood a specific expression of this altered function which becomes manifest upon transfer into the nonsensitive recipient".

In cell transfer studies in the guinea pig, Metaxas and Metaxas-Buehler (1955) found that recipients of cells from tuberculin-sensitive donors were no longer reactive to test 5 to 8 days after the transfer. They postulated that transferred sensitivity is directly mediated by the transferred cells and is lost as these cells die or are rejected.

In an elegant series of experiments in the guinea pig, Chase (1959) transferred competent living cells into normal recipients and into animals rendered specifically unresponsive to sensitization with the allergen employed (picryl chloride) by the prior feeding of the allergen in minute quantities. These animals were injected with the cells and the pattern of responsiveness of each individual animal was followed with respect to time. The sensitivity of these animals fell into two patterns. In all recipients, there was, of course, the initial period of hypersensitivity which appeared immediately after transfer of competent cells which lasted 5 to 8 days. In the majority of normal recipients, there appeared a second phase of hypersensitivity which persisted

for long periods, analogous to the findings in the work of Dr. Lawrence. In a few instances in the normal recipients, and almost invariably in the specifically unresponsive animals unless very large numbers of cells were transferred, the reactivity was monophasic, disappearing completely after one to two weeks. The suggestion was made that this second phase appeared to be the result of some form of active sensitization.

Experiments were undertaken here to extend these findings of Chase with the contact system and to determine whether the second phase of sensitivity in the guinea pigs was brought about by sensitization due to antigenic material in the transferred cells themselves or by the repeated testing (IV-A and Table 11).

Genetically unselected normal guinea pigs were used as recipients and, after transfer of small amounts of competent living cells, were divided into groups which received their initial contact tests at planned times after transfer. Some recipients were tested shortly after transfer (tests between T_0 and T_3) to establish the competence of the transferred cells, the mean reactivity of the group proving to be ' ++++ '. Other individuals receiving the same amounts of cells from the same cell pools were given their first contact tests at times ranging from T_{12} to T_{69}. Of 22 such recipients (Table 11) there was only one animal which gave a clearly positive reaction to contact test (recipient of 16.7×10^8 lymph node cells given intraperitoneally and tested first at T_{21}); the mean reaction of the late-tested group fell between ' 0 ' and ' $\pm$ '. From this result it can be concluded that, as was found by Chase and Metaxas and Metaxas-Buehler, sensitivity in non-inbred recipients of competent cells disappears within one to two weeks after transfer. Since sensitivity does not reappear, this tends to eliminate the possibility that the second phase of Chase's bimodal curve is the result of the presence of a special antigen contained in the transferred cells that leads spontaneously to active sensitization. In addition, it negates the hypothesis of transfer of self-perpetuating "immunologic information" in the guinea pig analogous to the interpretation suggested by Dr. Lawrence to explain the results in the human system.

Further, it was found that those recipients of competent living cells which reacted strongly to the initial early test, also reacted strongly to a second test made considerably later (T_{42} to T_{87}), the mean being ' +++ '.

As is seen clearly in Table 11, this level of reactivity to second contact test was markedly greater than that of normal control animals which were tested in parallel (mean, ' $\pm$ '). This result strongly supports the idea that the second phase of Chase's bimodal curve is due to sensitization by the contact testing, and in this case by a single test.[1] To explain the marked difference between normal animals and recipients of competent cells in repeated parallel testing, it is suggested that the initial test with allergen on recipients of living cells - in which early tests result in strong positive reaction and marked cellular infiltration often followed by tissue damage - may result in uptake of the allergen by the infiltrating cells and hence be more effective in initiating active sensitization. In the case of normal animals in which the allergen painted on the skin produces no initial inflammatory response, it would be likely that the allergen would not be as accesible to the immunologic system. Naturally, this is a relative matter, for only successive skin paintings can lead to active sensitization of normal 'toxicity control' animals in this degree.

A further comment is that this experiment clearly shows the dangers associated with repeated testing of normal animals or recipients of cellular material. In these experiments, one test made on recipients of live cells was sufficient to establish a sustained high level of hypersensitivity, and multiple testings do so on normal toxicity controls. Similarly, it does not seem impossible that repeated tuberculin testing of human subjects as used in Dr. Lawrence's studies could have, in some measure, induced an active and durable sensitization.

In summary, the transferred sensitivity of recipients of competent living cells to the chemical allergen appears to be of only short duration, probably existing only for the lifetime of the transferred cells. There was no evidence for the transfer of antigenic material within the living cells or for the transfer of any self-perpetuating "immunological information" from transferred cells to host cells. A single contact test on recipients of competent cells was shown to establish a second long lasting phase of hypersensitivity.

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[1] In earlier work, Chase and Battisto (1955) had written, "The persistence of sensitivity apparently is influenced by the very contact tests used for ascertaining its presence, for sensitivity appears to fall off when the initial testing of recipients (prepared in quadruplicate and tested at various times) is withheld for two or three weeks".

C. What is the Effect of Metabolic Inhibitors on the Transfer of Hypersensitivity by Treated Competent Living Cells?

When it became apparent that we were unable to transfer hypersensitivity by means of disrupted cells or cell extracts, means of inhibiting various cellular metabolic activities were sought in hopes of determining what biochemical functions were vital for the transfer of hypersensitivity.

In early experiments recounted in III-A, it was found that short exposures of competent cells to general metabolic inhibitors such as mercuric chloride or chloroform-saturated buffers rendered them non-viable (as determined by trypan blue staining) and incapable of transferring contact hypersensitivity.

In an effort to produce a more specific effect, competent lymph node cells were incubated in the presence of 2×10^{-3} M NaCN, since cyanide is well known to be a potent inhibitor of cytochrome oxidase in many mammalian cells. The results were surprising, namely, following cyanide treatment the cells appeared to be viable (nuclei showed no staining by trypan blue) and were fully competent to transfer contact hypersensitivity (IV-B and Appendix Table VI). Two possible explanations may be suggested to account for this result. It appears probable that lymphocytes derive most of their metabolic energy from glycolysis (Eisen et al. 1959) i.e. inhibition of their oxidative metabolism might not result in cell death. An alternative explanation is suggested by the very recent report of Karnovsky (1962) that leucocytes appear to have a cytochrome oxidase that is cyanide-insensitive.

Two relatively new antibiotics have created great interest by virtue of the fact that they appear to specifically affect DNA directly and not RNA. Shatkin et al. (1962) have shown that Mitomycin C, used in relatively low concentrations, has the ability to render mouse fibroblastic L-cells incapable of division in tissue culture, the cells remaining viable and capable of synthesizing RNA and protein. This technique serves as the basis for assay of this compound (Wakaki et al, 1958) since tumor cells are "sterilized" by treatment with small amounts of Mitomycin C and are unable to kill the test animals. In high concentrations, Mitomycin C was found to inhibit irreversibly DNA-dependent RNA synthesis and ultimately protein synthesis, but again the cells were able to survive for some time in culture. After six to eight hours

of incubation of the L-cells with high concentrations of Mitomycin C, even upon removal of the antibiotic, it was common thereafter to find nuclei disrupted, DNA depolymerized, and granules of DNA floating in the cytoplasm. The conclusion was reached that Mitomycin C acts directly to depolymerize DNA.

Actinomycin D is a compound which was found by Reich et al. (1962) to bind reversibly to DNA and to stop DNA-dependent RNA synthesis and ultimately to inhibit protein synthesis in mammalian cells in the culture and in bacteria. Indeed, tissue culture cells so blocked by Actinomycin allow intracellular multiplication of Mengovirus, a so-called RNA virus, whose DNA-independent RNA synthesis proceeds unaffected.

When competent lymphoid cells were incubated for 1.25 hours with Mitomycin C at 100μg/ml and with Actinomycin D at 12μg/ml,[1] there was complete abolition of the capacity of the cells to transfer contact or tuberculin hypersensitivity (Table 12), yet the cells were still viable (trypan blue test).

In contrast, when Mitomycin C was used at a lower concentration, 10μg/ml, the transfer capacity was hardly affected providing the cells were injected into the recipients by the intravenous route and providing the recipients were tested at T_0. However, when the cells were injected intraperitoneally and recipients not tested until T_3, only the weakest positive reactivity was seen, indicating that a concentration of 10μg/ml had had some effect on the cells.

Work is in progress with isotopically labelled precursors to study precisely the effects of the compounds under the conditions used on the transfer cells. If it can be shown that 10μg/ml Mitomycin C does inhibit the capacity of the cells to divide without significantly inhibiting RNA and protein synthesis, and that 100μg/ml Mitomycin C and Actinomycin D at 12μg/ml abolish RNA and protein synthesis, the following conclusions may be drawn:

(1) for the appearance of transferred hypersensitivity, continuing synthesis of RNA and/or protein by the competent cellls is required;

(2) cells rendered incapable of dividing but still able to synthesize RNA and protein for a time can transfer substantial levels of hypersensitivity,

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[1] These antibiotics were generously supplied by Dr. Leonard Hamilton, Sloan-Kettering Institute.

thus showing that the transferred cells are themselves directly involved in producing the reactivity in recipients and that "cloning" is not required;

(3) if it be assumed that the antibiotics used do not specifically inactivate a hypothetical "transfer factor", these results would support strongly the idea no sufficient amount of a "transfer factor" exists preformed within the transferred competent cells of the guinea pig. This follows from the fact that living competent cells, still metabolizing and containing all intracellular "factors", are unable to effect transfer after the metabolic inhibitors have acted on DNA, stopping RNA and protein synthesis. To assume that "transfer factor" is not specifically inhibited by these metabolic inhibitors is not unwarranted since the compounds are known to act only on DNA and since "transfer factor" obtained from human cells is reported to be unaffected by treatment with DNase (Lawrence, 1960) and is, therefore, presumably not polymerized DNA.

It thus appears that transfer of delayed-type hypersensitivity in the guinea pig is dependent on continued synthesis of RNA and/or protein and not on the quota of any preformed materials which the cells contain.

D. What is the Nature of the Specificity of Contact Reactions?

A great deal is known about the nature of the specificity of the immediate-type or antibody-mediated responses, mainly stemming from the classic work of Karl Landsteiner (summarized, 1945). The specificity of antibody reactions to the chemical compounds used as allergens in the present work was studied in detail by Chase (1947), by the introduction of a new and sensitive biological assay system for detecting small concentrations of antibody, since elaborated and now termed passive cutaneous anaphylaxis (PCA).

Less is known about the specificity of the delayed type hypersensitivities but the fact of specificity is apparent from practical tests (cf. Chase, 1959). It is clear that simple chemical compounds are capable of serving as antigenic determinant groups, but the resulting complexes that form and cause active sensitization are but little understood. Recent work has pointed to the surface of the carrier protein adjacent to the site of coupling as playing a large role in the specificity of contact reactions, such that the relative importance of the particular allergenic hapten has been questioned. It was important, therefore, to know how far such a principle would extend since we

needed a compound which could be used to detect non-specific reactivity (irritability) in recipients of disrupted cells and subcellular materials. Accordingly, guinea pigs actively and passively sensitized to picryl chloride and dinitrochlorobenzene were tested with a vriety of structurally distinct chemical allergens (Table 13). In testing for cross-reactivity, all of the test compounds with the exception of maleic anhydride were used in concentrations which produced slight irritative reactions on the skin of normal guinea pigs (toxicity controls) as shown on the first line of Table 13. In the "Intradermal" and "Adjuvant Methods" of sensitizing to picryl chloride, no significant cross-reactivity was found to o-chlorobenzoyl chloride despite the fact that its probable method of coupling with guinea pig protein is the same as picryl chloride which produced intensely positive reactions. When sensitization to picryl chloride was effected by the "Combination Method", in which skin painting with the simple chemical is superimposed on the "Adjuvant Method" (II-C), a broad degree of cross reactivity was present with all the test allergens, although of a far less order of intensity than with the specific allergen. In the case of percutaneous sensitization to dinitrochlorobenzene, no significant cross-reactivity was found, and even with use of the "Combination Method" only very slight cross reaction to paranitrosodimethylaniline was detectable.

Since cross reactivity can only parallel the specific, it would be expected that recipients of competent cells from donors sensitized to PCl by the "Combination Method" would produce cross reactions and that competent cells of DnCl-sensitive donors would not. For example, only those recipients of cells from PCl-sensitive donors (Combination Method) which were brilliantly positive to PCl at 1% showed any cross-reactivity with oClBCl at 15% (Figure 2) and conversely, as expected, recipients of cells from DnCl-sensitive donors (Combination Method) showed no cross reactions to oClBCl.

From these studies, accordingly, it was deemed suitable in recipients of cell extracts to employ oClBCl at 15% as a reagent to detect non-specific cutaneous irritability. The level of reactivity seen to picryl chloride in an occasional guinea pig -- be it recipient or normal control -- never exceeded ' $\pm\pm$ '. Obviously, were the reactions to picryl chloride ever regarded as truly specific, no cross reactivity to oClBCl could be discernable. In practice, it was found that reactivity to oClBCl at 15% was variable, probably detecting

only the most irritable skins, but even this approach was useful often; among the few recipients of cell extracts which showed weak reactivity to the specific allergen (Tables 8 and 9), most reacted as strongly or more so to the 15% oClBCl, thus indicating the reactions to be attributable to non-specific reactivity.

From the above, we see that cross-reactions in contact testing can be expected to occur on actively sensitized animals only where a very high level of specific hypersensitivity is present. It is to be noted that the sensitizations in question were effected only by direct autologous coupling of the allergenic chemical or (when mycobacterial adjuvant was used) with picrylated homologous erythrocyte stromata. In such animals, the presence of cross-reactions was discussed by Chase (1947):

> "With respect to cross-reactions seen in actively sensitized guinea pigs, it is evident that treatment of guinea pig skin with various simple substances, for sensitizing and for testing, will lead to the formation of corresponding conjugates all having in common a moiety, probably protein, with cavy specificity. Experience with artificial conjugated antigens, however, has shown the wisdom of selecting different protein components for the antigens to be used for testing and those used for immunizing in order to avoid reactions that depend upon structures other than the attached chemical radical. Such for instance would be areas of the protein surface having altered properties because of immediately contiguous sites of coupling, or spatial structures at the points of attachment, including the basal portion, but not necessarily the entirety, of the attached radical, together with the neighboring portions of the protein molecule. Cross-reactions to both these types of configuration rather than to the whole structure of the attached radical, have been described. (Landsteiner, 1945; pp 158, 159)".

Obviously, this is not the same situation which obtains in the specificity of antibodies directed against simple chemical haptens, in which the requisite for cross-reactivity was found to be close structural similarity of the test compound and the specific hapten (cf. Landsteiner, 1945). For example, structures of the chemical compounds tested here are sufficiently different that cross-reactions were not found with antibodies directed against the picryl or dinitrophenyl groups to conjugates of o-chlorobenzoyl chloride and phthalyl chloride (Chase, 1947).

> From some recent attempts to determine the basis of specificity of the delayed type reactions, other workers (Benacerraf and Levene, 1962; Gell and Silverstein, 1962; and Silverstein and Gell, 1962) concluded that the determinant of specificity in delayed-type

reactions is probably broader than those of antibody and can distinguish not only the haptenic group but also the type of linkage to the carrier.

In one experiment reported, guinea pigs were sensitized with highly picrylated guinea pig albumin (P-GPA) plus mycobacterial adjuvant. These animals were tested not by contact reactions to picryl chloride but by intradermal injections of various picryl-protein conjugates. A cross-reactivity observed upon intradermal injection of picrylated bovine serum albumin (P-BSA) was abolished by prior intraperitoneal injections of P-BSA ("desensitization"), leaving nearly intact hypersensitivity to the homologous P-GPA. Similar "desensitization" could not be secured by injecting intact BSA.

In another experiment, sensitized animals were tested intradermally by conjugates made with the same hapten but coupled to different groups on the same carrier protein (for example, nitrobenzene sulfonyl chloride reacting with free amino groups on guinea pig albumins produced a different conjugate than did diazotized nitraniline when coupled to the tyrosine and histidine groups of the carrier protein). Here cross-reactions did not occur even though the prosthetic grouping (nitrobenzene) and the protein were the same.

In another experiment, guinea pig albumin was coupled with different haptenic groupings and used to sensitize guinea pigs; cross-reactivity was found in varying degrees but to a much greater extent than we found in the contact system (Table 13).

Such observations are not incompatible with the findings presented here on contact reactions. In our system, we have a much greater degree of contact hypersensitivity to the specific chemical than to any of the cross-reacting compounds, as evidenced by stronger reactions to 0.006% picryl chloride than to 15% o-chlorobenzoyl chloride. The method of sensitizing with picrylated stromata in the presence of complete adjuvant certainly does not give rise to the high degree of cross-reactivity found by Gell and Silverstein when guinea pig albumin was conjugated and injected similarly. All of the allergenic compounds which gave cross reactions on animals sensitized to PCl by the "Combination Method", while differing structurally, nevertheless have in common a high reactivity toward amino groups and would be expected to react readily with skin proteins. Perhaps a simpler explanation for the wide spectrum of cross-reactivity than that favored by Gell and Silverstein would be that compounds which react with proteins in a similar way would alter or "denature" the proteins in similar ways. Thus, in addition to a hypersensitivity to the simple haptenic group, some component of hypersensitivity would be directed to "denatured protein", altered in the case of the cited experiments by *in vitro* coupling, and in the present case by *in vivo* coupling of the allergens to guinea pig skin proteins. This would obviate the need for, at this time, speculating that hapten-carrier linkages were "recognized" or that the "determinant sites"

were large. Clearly, haptens reacting with different groups on the same protein would be expected to "denature" the protein in different ways, and conversely, compounds which reacted with the same groupings would be expected to produce similar changes.

E. What is the Nature of the "Complete Antigen" in Contact Hypersensitivity?

Our interest was led from studies on the specificity of contact hypersensitivity to thoughts on the nature of the complete antigen which initiates skin reactivity. Proteins appear to be implicated in hypersensitivity reactions, yet in vitro-prepared conjugates of soluble proteins, even when used in mycobacterial adjuvants, have been rather ineffective as materials for sensitizing guinea pigs to high levels of contact reactivity.

As was presented in IV-D and Table 14, picryl chloride or dinitrofluorobenzene were coupled to a variety of materials and injected into guinea pigs. Some slight degree of contact reactivity was produced by the injections of small amounts of highly coupled bovine serum albumin in Freund's complete adjuvant as Benacerraf and Gell (1959b) have reported. In contrast, injection of picrylated or dinitrophenylated guinea pig erythrocyte stromata, (used routinely in this laboratory to sensitize animals in the "Combination" and "Adjuvant" methods) when injected in complete adjuvant produced very high levels of contact hypersensitivity to picryl chloride or dinitrochlorobenzene.

The effectiveness of the erythrocyte stromata as carrier in contrast to bovine serum albumin could be due to a number of factors. One possibility is that cell plasma membranes have some peculiar potentiality for initiating delayed-type hypersensitivity to haptens attached to them. To investigate this, a model for plasma membrane systems, myelin, was chosen. When calf brain myelin picrylated in vitro was injected into guinea pigs in mycobacterial adjuvant, an extraordinary degree of hypersensitivity to the first contact test with picryl chloride was found, equal to or greater than that resulting from the injection of coupled guinea pig stromata. When mycobacteria were not included in the injection mixture, picrylated myelin was ineffective in producing contact hypersensitivity. It is interesting to note thet the myelin used derives from a different species, i.e. it was a heterologous carrier.

A second possibility which occurred to us was that, while plasma membrane material was effective in sensitizing, perhaps the lipids contained

therein produced a non-specific adjuvant-like effect irrespective of the protein actually coupled. To examine this possibility, tubercle bacilli were picrylated *in vitro* and injected in incomplete adjuvant, since a lipid fraction of mycobacteria is thought to be responsible for sensitization to tuberculin (Raffel *et al.*, 1949). Contact tests with picryl chloride made at corresponding times, indicated that no sensitization had taken place. A further bit of evidence which tends to argue against this possibility is that a fraction from myelin, "proteolipid protein", which has been exhaustively extracted to remove all free lipids, when picrylated and injected in mycobacterial adjuvant produces as high a degree of sensitization as did the conjugated myelin.

It is to be noted that dinitrofluorobenzene-coupled stromata were almost as effective in our hands as picrylated stromata at producing contact hypersensitivity, whereas, using different carriers, Eisen (1959) and Gell and Benacerraf (1961b) found dinitrophenylated conjugates ineffective in producing dermal reactivity. (In both experiments, mycobacterial adjuvant was employed). The objection of Eisen (1959) that the successful sensitization with the conjugated stromata was due to uncoupled allergen dissolved in the lipid membrane may be discarded because the preparation of DNP-stromata used here had been stored in aqueous solution for seventeen years before use, and in that time any free dinitrofluorobenzene would have hydrolyzed.

The results of the experiments presented indicate that allergen-coupled plasma membrane material seems peculiarly effective in establishing high levels of contact hypersensitivity and suggests that it may represent what the immunologic system "sees" as the "complete antigen". The interest in this possibility is heightened by recent work on the nature of the homograft antigens by British workers (Haughton, 1962; Davies, 1962; Haughton and Davies, 1962). These investigators have found that "ghosts" prepared from mouse ascites tumor cells contain all the antigenic determinants possessed by the intact cells, including the antigens governed by the H-2 histoincompatability locus of the mouse. Further, autolytic digestion of the "ghosts" has produced soluble antigens rich in lipid and containing protein, binding specifically with antibody prepared against the whole tumor.

It is tempting to speculate that delayed-type hypersensitivity is peculiarly directed against altered cells and especially plasma membranes,

both in the contact and homograft systems. Possibly, hapten-coupled homologous albumins enjoy a superiority over other proteins in eliciting some cutaneous reactivity since they are easily adsorbed onto cell membranes. If the hypothesis that plasma membranes play an important role were verified, then a closer relation between contact hypersensitivity and the homograft system could be achieved. In addition, the justification of the use of the contact as a model for studying delayed-type hypersensitive responses would be strengthened, since in this instance, at least one among the whole mosaic of membrane antigens -- the attached chemical hapten -- would be known.

VI

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Plate I

SCALE OF CONTACT REACTIONS TO PICRYL CHLORIDE

One capillary drop 1% in olive oil, 24 hours previously.

VII

APPENDIX TABLES

ABBREVIATIONS AND SYMBOLS USED IN APPENDIX TABLES

Contact Reagents: (Details given in II-B and II-D)

PCl	picryl chloride
DnCl	dinitrochlorobenzene
oClBCl	o-chlorobenzoyl chloride
NDMA	p-nitrosodimethylaniline
PhthCl	phthalyl chloride
ethoxaz	2-phenyl-4-ethoxymethylene-5-oxazolone
Cantharadin	(used at 0.01% concentration in methyl cellosolve)
PPD	excipient-free purified protein derivative of tuberculin

Readings of Contact Reactions: See II-D and Plate I)

( )	indicates 48 hour test reading
[ ]	indicates 72 hour test reading

Readings of Tuberculin Reactions: (given in order of diameter, color, quality)

p	pink
pp	pale pink
fp	faint pink
vfp	very faint pink
c.	center (e.g., pp c.5)
bl.	blanched
br.	brownish
blu.	bluish cast
bry. ed.	brawny edema
ind.	indurated
al. fl.	almost flat
fl.	flat

T. C. refers to normal animals subjected to testing as 'toxicity controls'.

APPENDIX TABLE I-a

(DnCl 280)

Experiment: DnCl 280 Percutaneous Method Donors sacrificed on day 22, 5 days after last test.

Cell Source	Cell Pool	Recipient	Cells Used: Vol. (ml)	Cells Used: Count $\underline{n} \times 10^8$ ($\underline{n}$)	Cells Used: D/R	Transferred Material	Preparation of Material	Route Given	Contact Tests (with 1% dinitrochlorobenzene, except as indicated): T_2	T_3	T_4	oClBCl T_4	T_5	oClBCl T_5	T_6	oClBCl T_6
Perit.Exud.	30	1	0.90	----	6:1	Living Cells	Handled in G-H	i.p.	+++++	+++++	+++	++	++	++	++	+
		2	0.90	----	6:1	Sonicate+intact cells	Sonicated 3 min in G-H	i.p.	tr	tr	0	0	0	0	+	0
		3	1.35	----	9:1	Sonicate	Sonicated 6 min in G-H	i.p.	0	0	0	0	0	0	0	0
Spleen	30	5	0.53	----	6:1	Living Cells	Handled in G-H	i.p.	---	+++(++++)	++	tr	0	0	tr	0
		6	0.53	----	6:1	Sonicate+intact cells	Sonicated 2.5 min in G-H	i.p.	---	++(++)	+	tr	tr	+	tr	+
		7	0.80	----	9:1	Sonicate	Sonicated 5 min in G-H	i.p.	---	0	tr	0	0	0	0	0
		TC 1							tr	0	0	0	0	0	0	0
		TC 2							---	0	tr	0	0	0	0	0
Perit.Exud		$4Sp_1$	1.35	----	9:1	Sonicate	Same as Recip. 3	i.p.	++	++	++	tr	tr	+	+	+
Spleen		$8Sp_1$	0.80	----	9:1	Sonicate	Same as Recip. 7	i.p.	---	++(+)	++	tr	tr	tr	+	0
		TC $3Sp_1$							0	++	++	0	tr	0	0	0
		TC $4Sp_1$							---	+(++)	++	+	0	tr	+	+

Sp_1 - Tbc-induced peritoneal exudate present
0.5 mg Tbc injected in 0.5 ml paraffin oil 3 weeks previously.
0.001 mg Tbc in saline given i.p. 2 days before transfer.

APPENDIX TABLE I-b

(DnCl 283-A)

Experiment: DnCl 283-A Percutaneous Method Donors sacrificed on day 23, 6 days after last test

Cell Source	Cell Pool	Recipient	Cells Used: Vol. (ml)	Cells Used: Count $\underline{n} \times 10^8$ ($\underline{n}$)	Cells Used: D/R	Transferred Material	Preparation of Material	Route Given	Contact Tests (with 1% dinitrochlorobenzene, except as indicated): T_2	T_3	depil T_3	1/3% DnCl T_3	depil T_3	oClBCl T_3	T_4	oClBCl T_4	depil T_4	T_5
Perit.Exud.	22[a]	1	0.35	16.0	4:1	Living Cells	Handled in G-H.	i.p.	+++++	---	---	+(++)	++(+++)	±	++	±	+	---
		2[a]	0.13	6.0	1.5:1	Living Cells	Same as Recip. 1.	i.p.	+++++	---	---	±	++p	±	+++	0	0(+)	---
		3	0.53	23.8	6:1	Sonic. Supernatant	Sonicated 1 min in G-H. (2000 rpm-15')	i.p.	tr	tr	++	---	---	tr	±	+	++	tr
		4				Sonic Sediment		i.p.	tr	tr	++	---	---	tr	tr	0	±	0
		5	0.53	23.8	6:1	Sonic. Supernatant + $CHCl_3$-sat'd buffer treated Sediment	Same as Recip. 3&4 Sediment held 20 min in $CHCl_3$-sat'd G-H. Supernatant + sediment injected	i.p.	0	tr	+p	---	---	tr	tr	0	0	0
		6	0.53	23.8	6:1	Sonic. Supernatant	Sonicated 4 min in G-H (2000 rpm-15')	i.p.	0	++	±(++)	---	---	±	+	±	±	---
		7				Sonic. Sediment		i.p.	tr	±	++	---	---	++	±	±	+	---
Lymph Node		8	0.14	6.0	3:1	Living Cells	Same as Recip. 1.	i.p.	++p	+++p	+++p	---	---	---	+	±	0(+)	---
		9	0.43	18.2	9:1	Sonic. Sediment (50W)	Sonicated 10 min in 9kc, 50W Raytheon. (2000 rpm-15')	i.p.	tr	0	±	---	---	---	---	0	0	---
		10	0.26	10.7	5.4:1	Sonic.Supernatant(50W)	(4/10 supernatant lost)	i.p.	0	tr	+	---	---	---	---	±	+	---
		11	0.29	12.1	6:1	Sonic. Supernatant	Same as Recip. 3&4.	i.p.	0	tr	±	---	---	---	---	0	0	---
		12				Sonic. Sediment		i.p.	0	tr	++(++)	---	---	---	---	tr	0	---
		TC 1							0	±	+p	---	---	tr	±	tr	0(+)	---
		TC 2							0	±	++	---	---	±	+	±	0(++)	---
		TC 3							0	tr	+++p	---	---	---	tr	0	tr	---

a - Recipient 2 received 0.5:1 cells from pool and cells from 1 donor not included in pool.

APPENDIX TABLE II-a

(DnCl 283-B)

Experiment: DnCl 283-B Combination Method Donors sacrificed on day 50, 14 days after the last test

Cell Source	Cell Pool	Recipient	Cells Used: Vol. (ml)	Cells Used: Count n x 10^8 (n)	Cells Used: D/R	Transferred Material	Preparation of Material	Route Given	Contact Tests (with 1% dinitrochlorobenzene, except as indicated): T_2	depil T_2	oClBCl T_2	oClBCl depil T_2	T_3	depil T_3	oClBCl T_3	oClBCl depil T_3	cantharidin T_3	cantharidin depil T_3	T_4	cantharidin T_4	cantharidin depil T_4	T_5
Perit.Exud.	10	1	0.13	2.0	1:1	Living Cells	Handled in G-H	i.p.	+++	+++++	tr	tr	0	---	---	---	±±	±±	---	---	---	+++
		2	1.17	18.0	9:1	Sonic. Supernatant	Sonicated 10 min in G-H in 9kc, 50W Raytheon. (2000 rpm-15')	i.p.	tr	tr	tr(++)	++(+++)	0	---	---	---	++	++	---	---	---	0
		3				Sonic Sediment		i.p.	0	tr	0	tr	0	---	---	---	++	+++	---	---	---	0
Lymph Node	10	4	0.30	12.0	2:1	Living Cells	Handled in G-H	i.p.	tr	+p(++p)	0	tr	0	---	---	---	+	+	---	---	---	0
		5	1.20	52.0	8:1	Sonicate	Sonicated 3 min in G-H in 9kc, 50W Raytheon.	i.p.	tr	+	tr	±	0	---	---	---	+	±±	---	---	---	0
Spleen	10	6	0.24	6.6	2:1	Living Cells	Handled in G-H	i.p.	---	---	---	---	++(++)	+++++	tr	tr	---	---	+	+(++)	+++	---
		7	0.98	27.0	8:1	Sonicate	Sonicated 10 min in G-H in 9kc, 50W Raytheon	i.p.	---	---	---	---	tr	+p	+	+	---	---	±	+(++)	++(+++)	---
Normal Spleens	10	8	0.60	----	5:1	Living Cells	Handled in G-H	i.p.	0	±	0	±	0	---	---	---	±±	±±	---	---	0	0
		9	0.60	----	5:1	Living Cells	Handled in G-H	i.p.	0	±	±	±	0	---	---	---	+++	+++±	---	---	+	+
		TC 1							0	tr	0	tr	0	---	---	---	±	±	---	---	---	+
		TC 2							0	tr(±)	0	+	0	---	---	---	±(++)	0	---	---	0	0
		TC 3							tr	±	±	±	0	---	---	---	±±	±	---	---	---	0

APPENDIX TABLE II-b

(DnCl 284-A)

Experiment: DnCl 284-A Combination Method Donors sacrificed on day 44, 6 days after last test

Cell Source	Cell Pool	Recipient	Cells Used: Vol. (ml)	Cells Used: Count n x 10^8 (n)	Cells Used: D/R	Transferred Material	Preparation of Material	Route Given	Contact Tests (with 1% dinitrochlorobenzene, except as indicated): T_2	depil T_2	oClBCl T_2	oClBCl depil T_2	T_3	depil T_3	oClBCl T_3	oClBCl depil T_3
Perit.Exud.	20	1	0.30	5.2	2:1	Living Cells	Held in G-H until Recip.3-6 inj.	i.p.	tr	tr	0	tr	0	0	---	---
		2	0.07	1.3	0.5:1	Living Cells	Same as Recip. 1.	i.p.	±p	+++p	0	tr	tr	+	---	---
		3	1.28	22.2	8.5:1	Sonic. Supernatant	Sonicated 10 min in G-H in 9kc, 50W Raytheon. (1500 rpm-15')	i.p.	tr	tr	tr	tr(±)	0	0	---	---
		4				Sonic. Sediment		i.p.	0	0	0	0	0	tr	---	---
		5	1.28	22.2	8.5:1	Sonic. Supernatant	Same as Recip. 3	i.p.	0	tr(±p)	0	±	0	tr	---	---
		6				Sonic. Sediment	Same as Recip. 4	i.p.	0	±	0	0(tr)	0	tr	---	---
Lymph Node	20	7	0.81	25.5	6:1	Living Cells	Handled in G-H	i.p.	---	---	---	---	0	±(±±)	0	±
		8	0.27	8.5	2:1	Living Cells	Handled in G-H	i.p.	+p	±±(+++)	0	0	0	±±(+++)	---	---
		9	1.50	48.0	11.5:1	Sonic. Supernatant	Same as Recip. 3	i.p.	0	0	0	0	0	tr	---	---
		10				Sonic. Sediment	Same as Recip. 4	i.p.	0	0	0	0(±p)	0	tr	---	---
Spleen	20	11	0.32	9.6	2:1	Living Cells	Handled in G-H	i.p.	++++	+++++	0	0	0	±(+++)	---	---
		12	1.47	43.0	9:1	Homogenate Supernatant	Homogenized in 1% G-H.(1500 rpm-15') Sediment rehomogenized, supernatants pooled	i.p.	0	+p	0	0	0	0	---	---
		13				Homogenate Sediment		i.p.	0	tr(±p)	tr	tr(+p)	0	tr(+++p)	---	---
		14	1.47	43.0	9:1	Lyophilized Cells	Cells lyophilized as a pellet. Resuspended in cetane.	i.p.	---	---	---	---	±	---	---	---
Normal Perit.Exud.	6	15	0.60	4.6	6:1	Sonic. Supernatant	Same as Recip. 3.	i.p.	0	tr	tr	tr	0	0	---	---
		TC 1							0	0	0	tr(±)	0	tr(±±)	---	---
		TC 2							0	tr	tr	±p	0	tr(±)	---	---
		TC 3							0	tr	tr	+p	0	tr(+)	0	tr

APPENDIX TABLE III-a

(DnCl 284-B)

Experiment: DnCl 284-B Donors received Normal G.P. Stromata + Tbc. + Aquaphor, and were sacrificed on day 42, untested

Cell Source	Cell Pool	Recip-ient	Cells Used: Vol. (ml)	Cells Used: Count n x 10^8 (n)	Cells Used: D/R	Transferred Material	Preparation of Material	Route Given	Contact Tests (with 1% dinitrochlorobenzene, except as indicated): T_2	T_2 depil	NDMA T_3	OClBCl T_3	PhthCl T_3	Tuberculin Test PPD(25μg) T_{58}
Donors Tbn Sensi-tive only									0	tr(++p)	++	+	±	---
									0	tr(++p)	+++	tr	±	---
Perit.Exud.	11	1	0.39	----	3:1	Incompetent Live Cells	Handled in G-H.	i.p.	0	tr	+	tr	±	---
		2	1.04	----	8:1	Sonic. Supernatant	Sonicated 5 min in G-H. (1500 rpm-15')	i.p.	0(tr)	0(tr)	+	++	++	4,vfp,fl.
		3				Sonic. Sediment		i.p.	0	0	±	0	±	0
Spleen	12	4	0.52	----	4:1	Incompetent Live Cells	Handled in G-H	i.p.	0	tr	++	0	+	---
		5	1.04	----	8:1	Sonic. Supernatant	Sonicated 9 min in G-H (1500 rpm-15')	i.p.	0	0	++	tr	±	6,fl.
		6				Sonic. Sediment		i.p.	0	0	±	±	±	0
		TC 1							0	0	+	0	tr	---
		TC 2							0	0	+	0	0	---

APPENDIX TABLE III-b

(PCl 462)

Experiment: PCl 462 Combination Method Donors sacrificed on day 60, 4 days after last test.

Cell Source	Cell Pool	Recip-ient	Cells Used: Vol. (ml)	Cells Used: Count n x 10^8 (n)	Cells Used: D/R	Transferred Material	Preparation of Material	Route Given	Contact Tests (with 1% picryl chloride): T_1	T_2	T_3	Tuberculin Tests (50μg PPD): T_2	T_3	T_{25}
Perit.Exud.	14	1	0.15	---	1.5:1	Living Cells	Held 4 hours at 4° in S-H	i.p.	+ (++++)	++++	++++	11 vfp,c3	10 vfp,c.3	7.5 fp-,sl.el.
		2	0.55	---	6:1	Sonicate	Sonicated 9 min in S-H+RNA+Cys	i.p.	0 (±) [++++]	+++(++++)	++++	7x5 fp+,sl.th.	tr	5.5 fp,al.fl.
		3	0.55	---	6:1	Sonicate	Sonicated 5 min in 35% S-H	i.p.	0	0	0	5 spot	5x3 fp+,fl.	11 pp/p,sl.th.
Spleen	14	4	0.70	---	7:1	Frozen & Thawed Cells	Frozen at -68° in S-H+Cys. F.& T. 3x. Added 2mg. DNase+150mg $MgSO_4$- 16 min at 37°.	i.p.	tr	0	0	0	0	0
		5	0.70	---	7:1	Frozen & Thawed Cells	Frozen in S-C+NaF (0.02M.) F.& T. 3x. Diluted with S-H.	i.p.	0	0	0	7x4 tr color	0	6 fp,fl.

APPENDIX TABLE III-c

(PCl 463)

Experiment: PCl 463 Combination Method Donors sacrificed on day 43, 8 days after last test

Cell Source	Cell Pool	Recip-ient	Cells Used: Vol. (ml)	Cells Used: Count n x 10^8 (n)	Cells Used: D/R	Transferred Material	Preparation of Material	Route Given	Contact Tests (with 1% picryl chloride): T_2	T_3	T_5	Tuberculin Test (50μg PPD): T_2
Perit.Exud.	14	1	0.33	---	4:1	Sonicate	Sonicated 5 min in S-H+RNA+Cys	i.p.	0	0	---	11 blu flush,fl.
		2	0.83	---	10:1	Sonic. Supernatant	Same as Recip.1. (16,000rpm-75')	i.p.	0	0	---	11 diffuse,fl.
		3				Sonic. Sediment		i.p.	0	0	---	0
Lymph Node	13	4	0.70	---	7.5:1	Sonicate	Sonicated 10 min in S-H+RNA+Cys	i.p.	0	0	---	7 tr color,fl.
		5	0.60	---	5.5:1	Sonicate	Sonicated 10 min in S-H+Cys	i.p.	0	0	---	7 tr color,fl.
Spleen	13	6	1.20	---	6.5:1	Held Cells - Sonicate	Cells held 24 hours at 4°C. in S-H+RNA+Cys. Sonicated 7 min.	i.p.	0	0	---	9 blu flush
		7	1.20	---	6.5:1	Frozen & Thawed Cells	Frozen in S-H+RNA+Cys at -68°C, kept 3 days at -20°C - Thawed and injected into Recip. 6.	i.p.	---	---	0	---

APPENDIX TABLE IV-a

(PCl 464)

Experiment: PCl 464 Combination Method Donors sacrificed on day 45, 18 days after last test.

Cell Source	Cell Pool	Recip-ient	Cells Used: Vol. (ml)	Cells Used: Count n x 10^8 (n)	Cells Used: D/R	Transferred Material	Preparation of Material	Route Given	Contact Tests (with 1% picryl chloride): T_2	T_4	T_6
Perit.Exud.	12	1	0.59	---	5.5:1	Sonicate	Sonicated 8 min in S-H+Cys	i.p.	tr(±)	tr	++
		2	0.30	---	3:1	Sonicate	Same as Recip. 1.	i.p.	tr	0	±(++)
		3	0.20	---	2:1	Sonicate	Same as Recip. 1	i.p.	tr	0	+(+++)
		4	0.10	---	1:1	Sonicate	Same as Recip. 1	i.p.	0	0	+++
Lymph Node	12	5	0.36	---	3:1	Sonicate	Sonicated 6.5 min in S-H+Cys	i.p.	±(+)	tr	++++
		6	0.72	---	6:1	Sonic. Cell Debris	Same as Recip. 5. (2000rpm-10')	i.p.	0	0	tr(+)
		7				Sonic. Particulates	Sediment (Cell Debris) - Supernatant (100,000xg.-75')-Sediment (Particulates) and Supernatant	i.p.	0	tr	+++
		8				Sonic. Cell Sap	(Cell Sap)	i.p.	0	tr	++
		9	0.36	---	3:1	Sonicate	Same as Recip. 5. Held 3 hours	i.p.	0	0	±
Spleen	12	10	0.72	---	6.3:1	Held Cells - Sonicate	Cells held 18 hours at 0° in S-H+Cys. Sonicated 5 min.	i.p.	0	0	++
		11	0.63	---	5.7:1	Frozen & Thawed Cells	Held 19 hours in dry ice in S-H+Cys. Thawed, refrozen, thawed.	i.p.	±	0	+++
	TC 1								0	tr	++++±
	TC 2								0	tr	++(+++)
	TC 3								0	0	++

APPENDIX TABLE IV-b

(PCl 465)

Experiment: PCl 465 Combination Method Rockefeller Institute stock donors sacrificed on day 50, 6 days after test.

Cell Source	Cell Pool	Recip-ient	Cells Used: Vol. (ml)	Cells Used: Count n x 10^8 (n)	Cells Used: D/R	Transferred Material	Preparation of Material	Route Given	Contact Tests (with 1% picryl chloride): T_3	T_4	T_5
Perit.Exud.	9	1[a]	0.56	---	7:1	Sonicate	Sonicated 5 min in G-H+RNA+Cys	i.p.	0	0	0
		2[a]	0.12	---	1.5:1	Sonicate	Same as Recip. 1.	i.p.	tr	0	0
Lymph Node	10	3[a]	0.33	---	3:1	Living Cells	Handled in G-H	i.p.	tr	0	0
		4[a]	0.77	---	7:1	Sonicate	Sonicated 5.5 min in G-H+RNA+Cys	i.p.	0	0	---
Spleen	10	5[a]	0.39	---	3:1	Living Cells	Handled in G-H.	i.p.	±	tr	0
		6[a]	0.61	---	4.7:1	Sonicate	Sonicated 5 min in G-H+RNA+Cys at 17°	i.p.	tr	±	tr(++)
		7[a]	0.30	---	2.3:1	Sonicate	Sonicated 5 min in G-H+RNA+Cys at 40°C.	i.p.	tr	tr	+p
		TC 1[a]							tr	±p	+(++)

a - Hartley strain guinea pigs.

APPENDIX TABLE V-a

(PC1 466)

Experiment: PC1 466 Combination Method Donors Sacrificed on day 43, 3 days after last test.

Cell Source	Cell Pool	Recipient	Cells Used: Vol. (ml)	Cells Used: Count n x 10^8 (n)	Cells Used: D/R	Transferred Material	Preparation of Material	Route Given	Contact Tests (with 1% picryl chloride, except as indicated): T_1	T_2	T_3	T_3 1/3% PC1	T_5	T_5 1/3% PC1	T_6
Perit.Exud.	15	1	0.57	---	7.5:1	Sonicate	Sonicated 4 min in S-H+RNA+Cys	i.p.	---	tr	0	---	±	---	+++
		2	0.57	---	7.5:1	Sonicate	Sonicated 3 min in H+Sucrose	i.p.	---	0	0	---	tr	---	++
Lymph Node	15	3	0.50	---	4:1	Living Cells	Handled in S-H	i.p.	++(+++±)	++++	+++++	+++++	+++++	++	---
		4	0.25	---	2:1	Living Cells	Handled in S-H	i.p.	0(++++)	+++++	++++++	+++++	++++++	+++	---
		5	0.25	---	2:1	Living Cells	Handled in S-H	i.v.	+++++	+++++	+++++	+++++	+++++	+++++	---
		6	0.13	---	1:1	Living Cells	Handled in S-H	i.p.	±(++++)	+++++	+++++	+++++	+++++	+++++	---
		7	0.62	---	5:1	Sonicate	Sonicated 3 min in S-H+RNA+Cys	i.p.	---	0	0	---	tr	---	++
Spleen	15	8	0.45	---	2.5:1	Living Cells	Held 15 min in S-H	i.v.	+++(++++)	---	+++++	---	---	++	---
		9	0.46	---	2.5:1	Living Cells	Held 15 min in S-H+Cys (0.01M)	i.v.	±(+++)	---	++++±	---	---	±±	---
		10	0.43	---	2.4:1	Living Cells	Held 15 min in S-H+Cys (0.001M)	i.v.	++++	---	+++++	---	---	+++±±	---
		11	0.89	---	5:1	Sonicate	Sonicated 4.5 min in H+Sucrose	i.p.	---	±p	±p	---	±	---	+++
		TC 1							0	0	0	0	---	---	---
		TC 2							0	±(+)	±	±	---	---	---
		TC 3							---	0	---	---	±±	---	++++
		TC 4							---	tr	---	---	tr(±)	---	±±
		TC 5							---	tr	---	---	---	tr	±±p

APPENDIX TABLE V-b

(PC1 472)

Experiment: PC1 472 Combination Method Donors sacrificed on day 44, 7 days after last test.

Cell Source	Cell Pool	Recipient	Cells Used: Vol. (ml)	Cells Used: Count n x 10^8 (n)	Cells Used: D/R	Transferred Material	Preparation of Material	Route Given	Contact Tests (with 1% picryl chloride): T_o	$T_{1/4}$	T_1	T_2	T_3	T_4
Perit.Exud.	14	1	0.40	---	2:1	Living Cells	Handled in G-H. Injected immed.	i.p.	---	---	---	++++	++++	---
		2	0.58	---	3:1	Living Cells - Held	Held 6 hrs at room temp.in G-H	i.p.	---	---	---	+++++	+++	---
		3	0.58	---	3:1	Living Cells - Held	" " " " " " " "	i.p.	---	---	---	+++++	+++++	---
		4	0.58	---	3:1	Living Cells - Held	" " " " " " " G-S	i.p.	---	---	---	++++	±±	---
		5	0.58	---	3:1	Living Cells - Held	" " " " " " " "	i.p.	---	---	---	++++	+++++	---
Lymph Node	15	6	0.18	---	1:1	Living Cells	Handled in G-S-C.Injected immed.	i.p.	---	---	---	++++	++±	0(+)
		7	0.09	---	0.5:1	Living Cells	" " " " "	i.p.	---	---	---	±±	±	tr(±)
		8	0.73	---	4:1	Living Cells - Held	Held 6 hrs at 37°C. in Trowell's	i.p.	---	---	---	+++	++++	---
		9	0.73	---	4:1	Living Cells - Incubated 25 hrs.	Incubated 25 hrs at 37°C. in Trowell's Medium	i.p.	---	---	---	+++(++++)	±±	---
		10	0.91	---	5:1	Sonicate	Sonicated 4.5 min in G-S-C	i.p.	---	---	---	0	0	---
Spleen	15	11	0.33	---	1.5:1	Living Cells	Handled in G-S-C	i.p.	---	---	---	++	±±	tr(±)
		12	0.72	---	3.3:1	Sonicate (1 min)	Sonicated 1 min in G-S-C	i.p.	---	---	---	±(+++)	++(+++)	±
		13	0.72	---	3.3:1	Sonicate (4.5 min)	Sonicated 4.5 min in G-S-C	i.p.	---	---	---	tr	0	---
		14	0.72	---	3.3:1	Sonicate (5 min)	Sonicated 5 min in G-S	i.p.	0	0	0	0	0	---
		15	0.72	---	3.3:1	Sonicate (5 min)	Sonicated 5 min in G-S-C	i.p.	0	0	0	0	0	---
		TC 1							0	0	0	0	0	0
		TC 2							---	---	---	0	0	0
		TC 3							---	---	---	0	0	0

Experiment: PCl 467-A Combination Method Donors sacrificed on day 48, 9 days after last test.

Cell Source	Cell Pool	Recipient	Cells Used: Vol. (ml)	Cells Used: Count n x 10^8 (n)	Cells Used: D/R	Transferred Material	Preparation of Material	Route Given	Contact Tests (with 1% picryl chloride): T_2	T_3	T_4	Tuberculin Tests (50μg PPD): T_2	T_3
Perit.Exud.	22	1Sp*	0.82	---	5.5:1	Sonicate	Sonicated 4.5 min in G-H	i.p.	tr	0	---	9x6 vfp-,fl.	4 fp-,fl.
		2	0.82	---	5.5:1	Sonicate incubated and injected with normal exudate cells	Sonicated 4.5 min in G-H. Incubated 30 min at 37°C. with 0.64ml normal paraffin oil induced exudate cells.	i.p.	0	0	---	9 blu fp,al.fl	7 blu fp, fl.
		3	0.85	---	6:1	Rocked Cells - Supern.	Cells rocked 24 hrs in 12 ml. saline at 4°C. (1200rpm-5')	i.p.	0	0	---	9 blu fp,al.fl	0
		4				Rocked Cells-Sediment			0	0	---	0	0
Lymph Node	22	5	0.28	---	1.5:1	Living Cells	Handled in G-H	i.p.	+++±	+++	---	12x10 pp-,ind.	8 fp, sl.th.
		6	0.73	---	4.5:1	Living Cells - Treated with NaCN	Incubated 45 min at 37°C. with $2x10^{-3}$M NaCN in G-H.	i.p.	+++(++++)	+++++±	---	13 blu pp-,ind.	10 blu fp.sl.ind.
		7	0.75	---	5:1	Sonicate (4.5 min)	Sonicated 4.5 min in G-H	i.p.	tr	±	0	4.5 vfp, fl.	0
		8	0.75	---	5:1	Sonicate (1.5 min)	Sonicated 1.5 min in G-H	i.p.	+±	+±	+	5 vfp, fl. v	0
		9	0.75	---	5:1	Sonicate (1 min)	Sonicated 1 min in G-H	i.p.	tr	±	0	5.5 vfp, fl.	0
Spleen	22	10	0.68	---	4:1	Living Cells	Handled in G-H	i.p.	+±	+++	---	9 blu fp,sl.el.	6x5 fp, al.fl.
		11	0.68	---	4:1	Living Cells	Handled in G-S-C. Injected in S-C	i.p.	++++±	++++±	---	9 fp+,bry.ed.	6x5 vfp, fl.
		12	1.80	---	6.5:1	Living Cells incubated with PPD	Incubated 1.75 hrs at 37°C. in 50% fresh S-H+PPD (25μg).	i.p.	++++±	+++++	---	11.5 blu fp,ind.	12x10 p,w.ind.
		13			(3.9:1)	Supernatant Fluid	(1200rpm-5') 4/10 supern. lost		0	0	---	5 fp spot	0
		14	1.80	---	6.5:1	Sonicate with PPD	Sonicated 4.5 min in 50%S-H+PPD 25μg)	i.p.	0	±	---	9 blu fp,fl.	0
		TC 1							tr	tr	0	8 blu fp,fl.	0
		TC 2							tr	tr	---	8 fp,v.sl.el.	6 fp,al.fl.
		TC 3							---	---	---	---	6x4 fp-,al.fl.
		TC 4Sp*							0	tr	---	0	0
		TC 5Sp*							tr	tr	---	4vfp	8.5 vfp,fl.
												PCA Tests on Serum	
									T_{21}	T_{29}	T_{40}	T_{39}	T_{74}
Perit.Exud.		15	0.81	---	5.1	Sonicate emulsified in Freund's Adjuvant	Sonicated 4.5 min in 2.7ml G-H. Emulsified with 1.5 ml Complete Freund's Adjuvant.	i.m.	0	0	0	0	0
		TC 6							0	tr	0	0	0

*Sp - Animals injected 2 days before transfer with 1.5 ml sodium caseinate i.p. to produce a mononuclear exudate.

APPENDIX TABLE VII-a

(PCl 473-A)

Experiment: PCl 473-A Combination Method Donors sacrificed on day 44, 9 days after last test.

Cell Source	Cell Pool	Recipient	Cells Used: Vol. (ml)	Cells Used: Count $\underline{n} \times 10^8$ ($\underline{n}$)	Cells Used: D/R	Transferred Material	Preparation of Material	Route Given	Contact Tests (1% picryl chloride): T_2	Contact Tests (1% picryl chloride): T_3	Tuberculin Test (50μg PPD): T_2
Perit.Exud.	17	1	0.15	---	1:1	Living Cells	Handled in G-S-C	i.p.	++++	+++$\underline{+}$	8 blu,al.fl.
		2	0.56	---	4:1	Sonic. Supernatant	Sonicated 0.75 min in S-C (1100rpm-5')	i.p.	0	0	---
		3				Sonic. Sediment		i.p.	+	tr	6.5 fp, fl.
		4	0.56	---	4:1	Sonic. Supernatant + $HgCl_2$-treated Sediment	Same as Recips. 3, 4. Sediment held 2 hrs in $10^{-3}\underline{M}$ $HgCl_2$, separated and suspended in supern.	i.p.	tr	tr	0
		5	0.56	---	4:1	Sonic. Supernatant + $CHCl_3$-sat'd buffer treated Sediment	Same as Recip. 3, 4. Sediment held 20 min in $CHCl_3$-sat'd S-C	i.p.	0	0	---
		6	0.56	---	4:1	4 Successive Sonic Supernatants	Like Recip. 2. Sediment resonicated 0.75 min, etc. for 4 times. Sediment after 4 sonications (3 min) injected in G-S-C	i.p.	0	0	---
		7				Final Sediment		i.p.	0	0	---
Lumph Node	17	8	0.14	---	1:1	Living Cells	Handled in S-Trowell's Medium	i.p.	++	+	---
		9	0.07	---	0.5:1	Living Cells	" " " "	i.p.	0	0	---
		10	0.04	---	0.25:1	Living Cells	" " " "	i.p.	+	tr	---
Spleen	15	17	1.37	---	9.4:1	Lyophilized Cells	Sedimented from G-S-C. Plug frozen at -68°C. and lyophilized, resuspended in saline.	i.p.	tr	0	10 pp, v.sl.el.
		18	0.73	---	5:1	$HgCl_2$-treated Cells	Held 2 hrs at room temp. in saline + $HgCl_2$ ($10^{-3}\underline{M}$). Supernatant discarded, cells resuspended in saline.	i.p.	0	0	---
		TC 1							0	0	---
		TC 2							0	0	9 blu fp,al.fl.
		TC 3							tr	tr	7x2 fp furrow
		TC 4							0	0	10x9 fp+,v.sl.el
		TC 5							---	0	---

APPENDIX TABLE VII-b

(PCl 477)

Experiment: PCl 477 Combination Method Donors sacrificed on day 44, 6 days after last test.

Cell Source	Cell Pool	Recipient	Cells Used: Vol. (ml)	Cells Used: Count $\underline{n} \times 10^8$ ($\underline{n}$)	Cells Used: D/R	Transferred Material	Preparation of Material	Route Given	Contact Tests (with 1% picryl chloride, except as indicated): T_1	T_2	depil T_2	oClBCl T_2	oClBCl depil T_2	oClBCl T_4	Tuberculin Tests (25μg PPD): T_1	T_2	T_{21}
Perit.Exud.	20	1	0.28	8.3	2:1	Living Cells	Handled in G-H.	i.v.	++++$\underline{+}$	+++++	---	---	---	+++	16x14,pp+,sl.th.	---	10.5 p-,bry.ed.
		2	1.26	37.5	9:1	Sonicate (30°C.)	Sonicated 8 min in G-H+RNA (30°)	i.p.	---	---	---	---	---	---	---	---	0
		3	1.26	37.5	9:1	Sonic. (30°) Supern.	Same as Recip.2. (12,000rpm-15') Sediment washed once.	i.p.	---	0	$\underline{+}$	0	0	0	---	7x5 fp, sl.th.	0
		4				Sonic. (30°) Sediment		i.p.	---	$\underline{+}$	$\underline{+}$	0	tr(+p)	tr	---	5 tr color,fl.	8 tr color,fl.
Spleen	20	5	0.33	8.9	3:1	Living Cells	Handled in G-H	i.v.	++	++	---	---	---	++	12x11 fp,sl.th.	---	15x13 fp+,al.fl.
		6	0.94	25.1	8.5:1	Sonicate (30°)	Same as Recip. 2.	i.p.	---	tr	tr(+p)	0	0	0	---	5 vfp,fl.	8x8.5 vfp, fl.
		7	0.94	25.1	8.5:1	Sonic. (30°) Supern.	Same as Recip. 3.	i.p.	---	---	---	---	---	---	---	---	9 flush, fl.
		8				Sonic. (30°) Sediment	Same as Recip. 4.	i.p.	---	---	---	---	---	---	---	---	8 vfp, fl.
		TC 1							0	tr	---	---	---	+	7 fp-, fl.	---	9 fp, fl.
		TC 2							---	0	tr(+)	0	tr	0	---	4 vfp, fl.	10 vfp-,sl.el.
		TC 3							---	0(+p)	++p	0	0	0	---	0	12x10 pp,sl.el.
		TC 4							---	0	+p	0	0	0	---	0	5.5 tr color,fl.
		TC 5							---	---	---	---	---	---	---	---	0

APPENDIX TABLE VIII-a

(PCl 487-A)

Experiment: PCl 487-A Combination Method Donors sacrificed on day 37, 6 days after last test.

Cell Source	Cell Pool	Recip-ient	Cells Used: Vol. (ml)	Cells Used: Count n x 10^8 (n)	Cells Used: D/R	Transferred Material	Preparation of Material	Route Given	Contact tests (with 1% picryl chloride, except as indicated): T_o	T_1	oClBCl T_1
Perit.Exud.	16	1	0.15	2.6	1.5:1	Living Cells	Handled in G-H	i.v.	+++++	++++	0
		2	0.49	8.4	5:1	Sonicate (MSE)	Sonicated 7 min in G-H (MSE)	i.v.	±	tr	±
		3	0.30	5.1	3:1	Sonicate (MSE)	" " " " " "	i.v.	0	tr	0
		4	0.10	1.7	1:1	Sonicate (MSE)	" " " " " "	i.v.	0	0	0
		5	0.45	7.6	4.5:1	Frozen & Thawed Cells	F.& T. 10x in G-H+DNase(0.1mg/ml)	i.v.	tr	0	0
Lymph Node	16	6	0.17	9.5	1.5:1	Living Cells	Handled in G-H	i.v.	++++	±(++)	±
		7	0.41	24.0	3.7:1	Sonicate (MSE)	Sonicated 15 min in G-H (MSE)	i.v.	0	0	0
		8	0.33	19.5	3:1	Sonicate (MSE)	" " " " " "	i.v.	0	0	0
		9	0.16	9.5	1.5:1	Sonicate (MSE)	" " " " " "	i.v.	±	0	±
		10	0.06	3.3	0.5:1	Sonicate (MSE)	" " " " " "	i.v.	0	0	0
		11	0.55	32.5	5:1	Frozen & Thawed Cells	F.& T. 10x in G-H+Dnase(0.1mg/ml)	i.v.	±	±p	±
Spleen	16	12	0.26	5.6	1.5:1	Living Cells	Handled in G-H	i.v.	±(++)	++	0
		13	0.76	16.7	4.5:1	Sonicate (MSE)	Sonicated 15 min in G-H (MSE)	i.v.	0	0	0
		14	0.51	11.1	3:1	Sonicate (MSE)	" " " " " "	i.v.	tr	0	0
		15	0.26	5.6	1.5:1	Sonicate (MSE)	" " " " " "	i.v.	0	0	0
		16	0.86	18.5	5:1	Frozen & Thawed Cells	F.&T. 10x in G-H+DNase(0.1mg/ml)	i.v.	0	0	0
		TC 1							tr	0	0
		TC 2							tr	0	0
		TC 3							0	0	0

APPENDIX TABLE VIII-b

(PCl 491-A)

Experiment: PCl 491-A Combination Method Donors sacrificed on day 39, 5 days after last transfer.

Cell Source	Cell Pool	Recip-ient	Cells Used: Vol. (ml)	Cells Used: Count n x 10^8 (n)	Cells Used: D/R	Transferred Material	Preparation of Material	Route Given	Contact Tests (with 1% picryl chloride, except as indicated): T_o ‡‡	undepil half	depil half	oClBCl T_o	oClBCl undepil half	oClBCl depil half	T_2	oClBCl T_2	Intradermal Test (150 Picryl-GP serum.) T_o
Lymph Node	11	1	---	5.7	1.5:1	Living Cells	Handled in G-H	i.v.	+++++	---	---	±	---	---	+++	±	12 bp, m.th.
		2	---	5.7	1.5:1	Living Cells	Handled in G-H	i.v.	±±	(++)	(++++)	tr	(0)	(±)	±±	tr	6.5 fp, sl.el.
Spleen wbc*	11	3	---	3.9	3:1	Living Cells	Handled in G-H	i.v.	+++++	---	---	±	---	---	++++	++	17x15 bp,w.th.
L.N.Slices + Spleen wbc		4	---	---	8:1†	Cell Sap	Homogenized in Askonas Medium.	ip+iv	0	(0)	(±p)	0	(0)	(tr)	0	tr	---
		5	---	---		Microsomes	(5000xg.)-sediment discarded.	i.p.	0	(0)	(±)	0	(0)	(±p)	0	tr	---
		6	---	---	4:1†	Cell Sap	(100,000xg.-60') - supernatant	ip+iv	±	(±)	(±±p)	±	(+)	(±)	0	tr	7 fp+, al.fl.
		7				Microsomes	(Cell Sap) and sediment (Micro-	i.p.	0	---	---	0	---	---	0	0	7 vfp,fl.
		8	---	---	4:1†	Cell Sap	somes) collected.	ip+iv	tr	---	---	±	---	---	0	tr	---
		9				Microsomes		i.p.	0	(0)	(+)	0	(0)	(±p)	0	±	---
		TC 1							0	(0)	(±p)	±	(0)	(+)	0	±	9 vfp,al.fl.
		TC 2							tr	---	---	0	---	---	tr	tr	4 fp,al.fl.
		TC 3							0	(0)	(0)	tr	(0)	(0)	0	tr	6, fp,v.sl.el.
		TC 4							0	---	---	tr	---	---	tr	tr	4 vfp, al.fl.

* - rbc laked.

† - The ratio represents the combined number of lymph node donors and spleen donors.

‡‡ - The T_o test site was depilated over one-half the area after the 24 hr reading.
The post-depilated and normal halves were read separately at 48 hrs.

Experiment: PCl 487-B Combination Method Donors sacrificed on day 44, 13 days after last test

Cell Source	Cell Pool	Recipient	Cells Used			Transferred Material	Preparation of Material	Route Given	Contact Tests (with 1% picryl chloride, except as indicated)			
			Vol. (ml)	Count $\underline{n} \times 10^8$ ($\underline{n}$)	D/R				T_o	T_o	T_1	T_4
Spleen wbc*	10	1	0.37	7.1	1.5:1	Living Cells	Handled in G-H	i.v.	+++	tr	±(+)	tr
		2	0.49	9.5	2:1	Sonicate	Sonicated 7 min in G-H (MSE)	i.v.	tr	0	0	tr
		3	0.49	9.5	2:1	Sonicate	Same as Recip. 2	i.v.	0	0	0	tr
		4	0.98	19.0	4:1	Frozen & Thawed Cells	F.& T. 7x in G-H+DNase(0.1mg/ml)	i.p.	0	tr	tr	tr
		5	0.49	9.5	2:1	Frozen & Thawed Cells	Same as Recip. 4	i.v.	tr(±)	tr	tr	++
		6			~8:1	Sonicated Residual Splenic Stromata	Sonicated 11 min in G-H (MSE)	i.v.	0	tr	0	0
Lymph Node	10	7	0.23	7.6	1.5:1	Living Cells	Handled in G-H	i.v.	++++±	±	±(+++)	+++
		8	0.62	20.4	4:1	Sonicate	Sonicated 9 min in G-H (MSE)	i.v.	0	tr	0	tr
		9	0.28	9.2	1.8:1	Sonicate	Same as Recip. 8	i.v.	tr	tr	tr	+
		10	0.78	25.5	5:1	Frozen & Thawed Cells	F.& T. 7x in G-H+DNase(0.1mg/ml)	i.p.	0	±(+)	0	0
		11	0.31	10.2	2:1	Frozen & Thawed Cells	Same as Recip. 10	i.p.	±	0	0	tr
		12			~5:1	Supernatant of Sonic. Lymph Node Residue	Residue after teasing of cells sonicated 4 min in G-H (MSE) (1000rpm-8') Sediment re-sonicated 7 min in G-H (MSE)	i.v. + i.p.	0	tr	0	tr
		13			~8:1			i.p.	0	0	0	tr
Donors †	Sensitized to Tuberculin only											
Spleen	3	14	0.66	111	3:1	Frozen & Thawed Cells	Same as Recip. 4	i.p.	tr	tr	0	tr
Lymph Node	3	15	0.33	15.3	3:1	Sonicate	Sonicated 5 min in G-H (MSE)	i.v.	0	tr	0	++
		TC 1							0(±)	tr	0	±
		TC 2							0	±	0	++p
		TC 3							tr(+)	0	0(±)	++p
		TC 4							0	tr	0	0
		TC 5							0	tr	0	0

* - rbc laked

† - Donors received 1 mg Tbc + vaseline and paraffin oil, i.m., 52 days previously.

APPENDIX TABLE X-a

(PC1 492-A)

Experiment: PC1 492-A Combination Method Donors sacrificed on day 33, 6 days after last test

Cell Source	Cell Pool	Recipient	Cells Used: Vol. (ml)	Cells Used: Count $\underline{n} \times 10^8$ ($\underline{n}$)	Cells Used: D/R	Transferred Material	Preparation of Material	Route Given	Contact Tests (with 1% picryl chloride, except as indicated): T_o ‡	undepil half	depil half	oC1BCl T_o
Lymph Node	3	1	---	7.2	2:1	Living Cells	Handled in G-H	i.v.	++++	(++++)	(+++++)	tr(+)
		2	---	3.6	1:1	Living Cells	Handled in G-H	i.v.	++	(++++)	(+++++)	+(++)
Spleen wbc*	3	3	---	6.0	2:1	Living Cells	Handled in G-H	i.v.	++++	(+++)	(++++±)	+
		4	---	3.0	1:1	Living Cells	Handled in G-H	i.v.	++++±	(++++±)	(+++++)	+
L.N.Slices + Spleen wbc	8 + 8	5	---	---	5.4:1 †	Cell Sap	Homogenized in Askonas-Hanks (Turk) - (5000rpm-15') supernatants pooled; sediment rehomogenized and supernatant kept separate; sediment discarded (100,000xg.-60')-supernatant (Cell Sap) and pooled pellet material (Microsomes) injected.	i.v.	tr	(0)	(tr)	0(±)
		6	---	---	5.4:1 †	Cell Sap		i.v.	tr(±)	(0)	(±)	0
		7	---	---	5.4:1 †	Cell Sap		i.v.	0	(0)	(0)	0
		8				Cell Sap from rehomogenized sediment		i.v	tr	(tr)	(tr)	0
		9			5.4:1 †	Microsomes		i.p.	0	(0)	(0)	0
		10			5.4:1 †	Microsomes		i.p.	tr	(±p)	(++p)	0
		11			5.4:1 †	Microsomes		i.p.	±	(++)	(++)	+(++)
		TC 1							++	(±)	(±)	++
		TC 2							+	(+p)	(++p)	±
		TC 3							tr	(0)	(0)	0
		TC 4							tr	(0)	(++)	tr
		TC 5							±	(tr)	(±)	++

* - rbc laked.

† - The ratio represents the combined number of lymph node donors and spleen donors.

‡ - The T_o test site was depilated over one-half the area after the 24 hr reading.
The post-depilated and normal halfs were read separately at 48 hrs.

APPENDIX TABLE X-b

(PC1 492-B)

Experiment: PC1 492-B Combination Method Donors sacrificed on day 35, 8 days after last test

Cell Source	Cell Pool	Recipient	Cells Used: Vol. (ml)	Cells Used: Count $\underline{n} \times 10^8$ ($\underline{n}$)	Cells Used: D/R	Transferred Material	Preparation of Material	Route Given	Contact Tests (with 1% picryl chloride, except as indicated): $T_{-1/4}$	oC1BCl $T_{-1/4}$	T_o	oC1BCl T_o	T_2	oC1BCl T_2
L.N.Slices + Spleen wbc*	10(RI)	1(H)	---	---	6.6:1†	Cell Sap	Homogenized in Askonas Medium Sedimented (1100rpm-5') Supernatant - 100,000xg-60') Cell Sap and Microsomes Sediment - rehomogenized (100,000xg-60') Residue Cell Sap and Residue Microsomes	i.v.	---	---	0	0	0	0
		2(RI)	---	---	6.6:1†	Cell Sap		i.v.	tr(±)	±	tr	++	---	---
		3(RI)	---	---	6.6:1†	Cell Sap		i.v.	tr	±	tr	+	+	tr
		4(H)	---	---	6.6:1†	Microsomes		i.p.	---	---	tr	tr	0(±)	±(++)
		5(RI)	---	---	6.6:1†	Microsomes		i.p.	tr	+	tr	+	---	---
		6(RI)	---	---	6.6:1†	Microsomes		i.p.	---	---	tr	0	tr	0(±)
		7(RI)	---	---	---	Residue Cell Sap		i.v.	---	---	tr	tr	0	0
		8(RI)	---	---	---	Residue Microsomes		i.p.	---	---	0	0	0(±)	+p(+p)
		TC 1							---	---	tr	tr	0	0
		TC 2							---	---	0	0	0	0
		TC 3							0	±	0	±	---	---
		TC 4							tr	±	0	tr	---	---
		TC 5							---	---	0	±	tr	±
		TC 6							---	---	±p	++p	±p	++p
		TC 7							---	---	tr	tr	++p	tr
		TC 8							---	---	tr	±	+p(++p)	+(++)

Footnotes as in PC1 492-A above. (RI) - Rockefeller Institute stock guinea pigs. (H) - Hartley stock guinea pigs.

Experiment: Tb 145. Donors sensitized by injection of 1.5mg Tbc. in p.o. + vaseline, and sacrificed at day 14.

Cell Source	Cell Pool	Recipient	Cells Used: Vol. (ml)	Cells Used: Count $\underline{n} \times 10^8$ ($\underline{n}$)	Cells Used: D/R	Transferred Material	Preparation of Material	Route Given	Tuberculin Tests (25μg PPD): T_2	Tuberculin Tests (25μg PPD): T_3
Perit.Exud.	18	1	0.65	9.0	6:1	Living Cells, incub.	Cells incub. 2hrs at 37° in 12ml S-E* - (1100rpm-5') - cells washed in G-H	i.p.	11.5 fp, c.5pp, bl.c.2, sl/m ind.	11 fp/pp, pp c.7, mod ind.
		2	0.65	9.0	6:1	Living Cells incub. with PPD	Cells incub. 2hrs at 37° in 12ml S-E + PPD (3.3μg/ml)- (1100rpm-5') - Cells washed in G-H	i.p.	10 fp, al. fl.	12 fp, sl/mod ind.
		3				Supernatant	Supernatant clarified	i.p.	spot	flush over 7.5,al.fl.
		4	0.65	9.0	6:1	Living Cells incub. with O.T. in sac.	Cells incub. 3hrs at 37° in 14ml S-E + O.T.(1:200) inside dialysis sac against 42ml S-E outside. Cells washed in G-H	i.p.	11 fp, pp c.5,ind.	12 pp,bl.c.4, ind.
		5				Inner Sac Fluid (supernatant)	Inner fluid (cell supern.)	i.p.	spot	flush over 7, fp c.4, al fl.
		6				Outer Fluid	Outer fluid (dialysate)	i.p.	5 colorless,al.fl.	6 blu fp, tr.el.
Lymph Node	18	7	0.31	10.3	9:1	Living Cells, incub.	Same as Recip. 1.	i.p.	9 vfp, fp c.4,fl	12.5 fp/pp+, m.ind.
		8	0.31	10.3	9:1	Living Cells incub. with Old Tuberculin	Cells incub. 2hrs at 37° in 12ml S-E + O.T.(1:200) - (1100rpm-5') - Cells washed in G-H	i.p.	11.5 fp/pp, ind.	12.5 pp, v.m.ind.
		9				Supernatant	Supernatant clarified	i.p.	prac 0	4 fp, al.fl.
Toxicity Controls		TC 1Sp					Injected with 78ml S-E (3 injs)	i.p.	spot	4 vfp-, fl
		TC 2Sp					Injected with PPD (100μg)14ml S-E	i.p.	4 y, al fl.	spot
		TC 3Sp					Injected with O.T. (0.56ml of 1:2 O.T. in 28ml S-E)	i.p.	v.sl. blu, 6,fl.	6 vfp, tr el.
		TC 4							dot	8 vfp--, al.fl.
		TC 5							spot	spot
		TC 6							dot	dot

* S-E refers to a fortified Eagle's medium (Gomatos et al., 1962) containing 5% newborn calf serum.

End