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Nuclear and Plasma Membrane Properties of Macrophage Heterokaryons and Hybrids

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NUCLEAR AND PLASMA MEMBRANE PROPERTIES OF MACROPHAGE HETEROKARYONS AND HYBRIDS

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SUMMARY

Mouse peritoneal macrophages, which do not synthesize DNA in vitro, were fused with a strain of mouse melanoma cells which proliferates rapidly in vitro. The plasma membrane of the macrophage has specific receptors which enable the cell to ingest antibody-coated sheep red cells and is also rich in a divalent cation dependent adenosine triphosphatase (ATPase) activity. The melanoma cell lacks these macrophage membrane markers.

High yields of mouse macrophage-melanoma cell heterokaryons and macrophage-macrophage homokaryons were obtained through the Sendai virus-induced fusion of cells spread on a glass surface. After fusion there was a striking reorganization of cellular architecture by means of a colcemid-sensitive process. Heterokaryons were isolated through the use of differential trypsinization and many underwent division to form melanoma-like hybrids. The selective uptake of dextran sulfate by macrophages served as a useful cytoplasmic marker in identifying hybrids.

Many characteristic macrophage properties were altered in the heterokaryons. Within an hour of fusion macrophage nuclei became swollen, nucleoli were more prominent and increased nuclear RNA synthesis occurred. Two - three hours after fusion a wave of DNA synthesis took place in the previously dormant macrophage nuclei. DNA synthesis was induced in macrophage nuclei irrespective of the number of macrophage nuclei present per melanoma nucleus in each heterokaryon. Fifty to 80% of macrophage nuclei initiated DNA synthesis in the three - seven hour period after fusion. The activation of most 11 - 12 day chick red cell nuclei in melanoma cytoplasm took longer than ten hours. The lag before DNA synthesis may reflect

the heterochromatin content of each nucleus.

Studies with actinomycin showed that heterokaryon RNA synthesis was essential for subsequent macrophage DNA synthesis. This RNA was synthesized one - four hours before the DNA and was unlikely to be ribosomal RNA since it was insensitive to < 0.1 μg/ml actinomycin.

Melanoma cells and macrophages were treated before fusion with actinomycin and bromotubercidin to bring about a more selective inhibition of RNA synthesis. Macrophages pretreated for one hour with 5 μg/ml actinomycin showed less than 20% of control RNA synthesis in the first four hours after fusion, but a normal activation of macrophage DNA synthesis. Pretreatment of melanoma cells for three - seven hours with 5 μg/ml bromotubercidin, a reversible inhibitor of RNA synthesis, prevented macrophage DNA synthesis without affecting macrophage RNA synthesis in the heterokaryon (81% of control). These studies showed that only melanoma RNA synthesis was essential for the production of macrophage DNA.

The exposure of one cell partner to actinomycin before fusion caused cross-toxicity of the untreated nucleus after fusion. Bromotubercidin, an adenosine analogue which is incorporated into RNA, did not give rise to such cross-toxicity after fusion. Once the macrophage nucleus is activated in the heterokaryon it becomes less sensitive to the action of actinomycin.

Cycloheximide treatment (1-5 μg/ml) of heterokaryons during the preceding lag period inhibited the initiation of macrophage DNA synthesis in a reversible fashion. Each type of cell was also treated with streptovitacin A, an irreversible inhibitor of protein synthesis. Pretreatment of the melanoma cells (1-2 μg/ml), one hour before

fusion, inhibited the induction of macrophage DNA synthesis in heterokaryons, whereas pretreatment of macrophages had no effect. Melanoma cell pretreatment reduced the incorporation of ^{3}H leucine into the cytoplasm and nuclei of heterokaryons, whereas macrophage pretreatment had no effect. These experiments suggested that melanoma proteins played an important role in the initiation of macrophage DNA synthesis.

The relationship between the melanoma cell cycle and macrophage DNA synthesis was studied with synchronous melanoma cells. If the melanoma cells were in S phase at the time of fusion, macrophage DNA synthesis occurred two hours later. However, the fusion of melanoma cells in G_1 delayed macrophage DNA synthesis until the melanoma cells had entered S. Experiments with actinomycin and cycloheximide showed that RNA and protein, essential to achieve DNA synthesis in the macrophage nucleus, were made during late G_1 as well as S.

Melanoma cells and macrophages differ in their radiolabeled acid-soluble products after incubation in ^{3}H thymidine. Thymidine taken up by the macrophage remained unphosphorylated, whereas it was recovered mainly as thymidine triphosphate from melanoma cells.

These findings suggest that the melanoma cell provides the RNA, protein and precursors which initiate macrophage DNA synthesis. In the absence of a requirement for new macrophage RNA and protein synthesis, other changes must be responsible for the two - three hour delay in DNA synthesis. These may involve physical changes in DNA, associated with swelling, as well as the transport of melanoma products into the macrophage nucleus.

The fate of the macrophage membrane markers was examined in both heterokaryons and homokaryons. Macrophage homokaryons con-

tinued to exhibit active phagocytosis of sensitized erythrocytes. The phagocytic receptor could be detected in heterokaryons shortly after fusion, but was progressively lost, at an exponential rate, over the next 12 - 24 hours. Another macrophage surface marker, the ATPase, could be demonstrated histochemically on heterokaryons. Shortly after fusion, it was present in discrete regions, but it became more diffuse and disappeared within a day.

Exposure of heterokaryons to trypsin (1-100 μg/ml/30'/37°C) results in the reappearance of initial receptor activity and the unmasking of the surface receptor. This property is again lost upon subsequent cultivation. The masking process takes place when cells are cultivated in the absence of IgG so that the adsorption of antibody from the medium is not responsible for this phenomenon. Inhibition of heterokaryon protein synthesis preserves phagocytic activity in a reversible fashion and prevents the masking of macrophage receptors. Inhibition of melanoma RNA synthesis before fusion is also able to block subsequent masking, but is ineffective if delayed until after fusion. U-v irradiation of the melanoma cell before fusion prevents subsequent masking, whereas similar treatment of the macrophage has no effect.

Antibody coated red cells attach diffusely to the surface of unmasked heterokaryons, suggesting that the macrophage receptor has become distributed over the whole cell surface. The macrophage receptor undergoes a change in function soon after fusion with a melanoma cell, since a higher concentration of antiserum is necessary to bind red cells to the heterokaryon. This change is independent of heterokaryon protein synthesis.

These results suggest that heterokaryon formation causes an early spreading and mixing process of some membrane components, as well as a slower masking process. Melanoma RNA synthesis re-

sults in the production of membrane protein which is responsible for the masking phenomenon, thus illustrating a novel mechanism for altering the expression of plasma membrane properties.

The question of macrophage receptor synthesis was investigated in DBA/2 mouse macrophage x mouse LMTK$^-$ cell hybrids. Three clones and one mass culture were isolated by their ability to grow in HAT selection medium. These hybrids retained 85 - 100% of the sum of their parental chromosomes and expressed genes derived from both parent cells, including glucose phosphate isomerase isozymes and H-2 antigens. The hybrids displayed ATPase activity intermediate between that of the macrophage and L cells. The macrophage-specific receptors for antibody coated red cells or complement could not be demonstrated on hybrid cells. It is unlikely that the selective absence of these receptors was due to loss of the appropriate genes.

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CHAPTER I

A. General Introduction

Hybrid cells were first produced by chance. In 1960 Barski, Sorieul and Cornefert (1960) found cells of hybrid karyotype in a mixed culture of mouse cells. The significance of this observation was readily grasped by Ephrussi who, along with other geneticists like Pontecorvo (1956) and Lederberg (1958), was alert to the opportunities which parasexual cell mating offered for genetic analysis in animal cells. Ephrussi and his coworkers (1962, 1964) repeated the observation and initiated a series of experiments to study the chromosome constitution and phenotypic properties of hybrids made between different mouse cells, as well as between mouse and rat cells. In their hands cell hybridisation was an uncontrolled, apparently spontaneous, event which occurred rather rarely when different cells were simply mixed and grown together in tissue culture. Although hybrids arose with only low frequency, it became possible to isolate the hybrid cells by using a selection system devised by Littlefield (1964). This medium contained hypoxanthine, aminopterin and thymidine, (HAT), and permitted the hybrid cells to multiply in vitro while the appropriate parent cells died.

In 1965 Harris and Watkins showed that Sendai virus could be used as an agent to promote cell fusion for the purpose of genetic studies. Previous work, done in several laboratories, had shown that certain viruses were able to induce giant cell formation in tissue culture (Enders and Peebles, 1954; Henle et al, 1954; Chanock, 1956; Marston, 1958; Okada, 1958). Harris and Watkins took advantage of an important observation, which had been made by Okada and Tadokoro (1962), that ultraviolet irradiation could destroy the infectivity of Sendai virus

without inactivating its fusing properties. This eliminated the problem of virus growth in the fused cells and made it feasible to use virus-induced cell fusion as an experimental tool. Since receptors for Sendai virus exist on a wide variety of animal cells, including those of many species (Andrewes, 1964), it now became possible to control this technique and apply it to almost any cell, including differentiated cells which do not divide in vitro. Virus fused cells can also give rise to proliferating hybrid cell lines (Yerganian and Nell, 1966) so that the methods were now available to initiate rapid growth in the new science of animal somatic cell genetics.

The technique of cell fusion has already made it possible to ask a variety of unusual question in experimental cell biology. It is my purpose to review the principles behind this novel research tool, based on the natural history of fused cells, and to illustrate the range of biologic problems to which this technique has already been applied. The main technical problems with this method have apparently been solved during the past decade and it is likely that somatic cell hybridisation will be widely applied in the future. This is an appropriate time to emphasise some of the hidden assumptions which still call for considerable caution in future experiments.

B. The Role of Sendai Virus

In addition to the reasons already mentioned, Harris and his associates (1966) chose Sendai virus for their fusion experiments because it is safe and relatively easy to handle. It shares the ability to fuse cells with other members of the para-influenza group of myxoviruses, for example Simian virus 5 (SV-5), and most of the information to be discussed below applies to all the viruses of this group (Andrewes, 1966). Herpes simplex, and other unrelated

viruses are also able to fuse cells (Roizman, 1962).

Sendai, like other paramyxoviruses, has a helical nucleocapsid, containing a single-stranded RNA genome (Blair and Duesberg, 1970), and a lipoprotein envelope which is responsible for the fusing property (Okada, 1969). These viruses mature by a remarkable budding process which has been studied in considerable detail by Choppin, Compans and Holmes at this University (Compans and Choppin, in press). The newly synthesised viral nucleocapsid becomes aligned beneath a patch of cell membrane which apparently contains viral genome determined proteins, and lipids derived from the host cell (Choppin et al, in press). The nucleocapsid is wrapped in a membrane envelope and shed from the cell in the form of spiked, pleomorphic viral particles. The envelope of the virus carries the strain specific antigens, neuraminidase, hemagglutinin and a hemolysin. The hemolysin is closely related to its fusing property (Holmes, 1968). The hemagglutinin is clearly distinct from the fusion factor and is therefore an unreliable indicator of fusing potency.

Susceptible cells can be fused in two ways. The first is by exposing them to high multiplicities of non-infectious virus, in which case fusion proceeds rapidly, "from without". The second is by infection with low multiplicities of virus, in which event fusion "from within" is delayed till after replication (Okada, 1969; Gorbunova, et al 1963; Cascardo and Karzon, 1965; Holmes and Choppin, 1966). The Sendai virus can be inactivated by treatment with ultraviolet light or β-propiolactone (Neff and Enders, 1968).

In spite of much effort, the detailed mechanism of fusion has remained elusive. Okada, who has contributed much to our understanding of this process, concluded that several stages are involved (Okada et al, 1966) First, the virus particles become attached to

cells by a temperature independent step which often results in cell clumping. Second, the virus exerts a lytic effect on the cell membrane. This resembles an enzyme action in its dependence on temperature (best at 37°C) and optimal pH (7.3 - 7.8) (Okada, 1958; Okada, 1962). Third, cell membranes may coalesce as adjacent cells attempt to repair this lesion. This step requires energy, which may be provided in the form of a nucleoside triphosphate (Okada et al, 1966), and Ca^{++} ions (Okada, 1966). No nucleic acid or protein synthesis is necessary for Sendai virus to fuse cells (Okada, 1969; Steplewski et al, 1969). Once a membrane bridge forms, the cytoplasm of the two fused cells intermingles.

Virologists have made strenuous but unsuccessful efforts to isolate a fusing "enzyme" or factor from these viruses. Indeed, several studies suggest that the viral coat has to be intact to bring about fusion and any treatment which disrupts the lipoprotein envelope of the virion apparently destroys this activity (Kohn, 1966; Kohn and Klibansky, 1967; Holmes, 1968).

Ultrastructural studies have not helped to settle the question of the fusion mechanism. Howe, Morgan and other workers claim that the virus envelope can fuse with the cell membrane and that free nucleocapsid is then released into the cytoplasm (Morgan and Howe, 1968; Hosaka and Koshi, 1968; Howe and Morgan, 1968). This resembles the reverse of the budding process and may facilitate cell fusion. The morphologic evidence for this theory is in dispute, especially since a proportion of adsorbed virus enters cells inside phagocytic vacuoles (Dales, 1965; Holmes, 1968).

The geneticist who wishes to use virus as a fusing agent soon finds that cells vary considerably in their "fusability"; for instance,

certain baby hamster kidney fibroblasts form huge syncytia after treatment with SV-5 (Holmes, 1968), whereas polymorphonuclear leukocytes cannot be fused (Okada, 1969). It is possible that any step in the sequence outlined above may be responsible for the failure to fuse. In addition, the ability of cells to form homokaryons and heterokaryons varies considerably.

The different response of each cell to fusing virus must ultimately depend on the chemical structure and organisation of the cell membrane and the virus envelope. In recent studies Choppin and his coworkers (1971, in press) compared the chemical composition of several paramyxoviruses and of cell types which differ in their degree of fusion as well as their yield of virus particles. The viral nucleocapsid protein, two coat glycoproteins, the neuraminidase and hemagglutinin, and two other proteins have been identified by electrophoresis of purified virions in polyacrylamide gels. Whereas these viral proteins differ completely from those of the cell membrane, the cell membrane lipids seem to be quantitatively incorporated into the virus envelope, with only minor exceptions. Moreover, the presence of cellular lipids containing a high ganglioside content correlated with high fusion efficiency. In contrast, a high phosphatidyl ethanolamine content correlated with a high yield of infectious virus particles and poor fusion. These interesting observations suggest that the lipid composition of the cell plasma membrane does influence the response of different cells to paramyxoviruses.

The use of Sendai virus as fusing agent is not without risk. The propiolactone treatment, introduced by Neff and Enders (1968), may abolish viral infectivity more effectively than ultraviolet irradiation. The possibility of persistent low grade infection in culture should always be borne in mind since it is known that cell lines may carry

myxoviruses without any morphologic effects (Walker, 1968). The presence of such virus is especially undesirable in longterm, hybrid cultures since possible viral-induced chromosome breakages may interfere with genetic analysis (Nichols et al, 1964). There are other complications associated with the use of high viral multiplicities. These include cytotoxic effects, inhibition of RNA synthesis (Ghendon et al, 1969) and depression of mitotic activity (Harris, 1970). In the final analysis it would no doubt be desirable to use a defined chemical moiety for cell fusion, rather than a whole virus particle. Perhaps the recent advances in characterising the macromolecular constituents of these viruses will still make it possible to identify and isolate such a fusion factor for future use.

C. The Properties of Heterokaryons and Homokaryons

A striking conclusion which followed from the early work of Harris and his associates (1966) was that heterokaryons formed from cells of widely divergent species were viable and that there was no intrinsic incompatibility between different cell types. Since then studies in a variety of heterokaryon systems have shown that subtle interrelationships between nuclei, cytoplasm and membrane may occur in fused cells. I want to review the principal findings of these studies.

1. Cell organization. Cell fusion has not been exploited often enough to study the architecture of animal cells. The only detailed study, to date, is the work of Holmes and Choppin (1968) at this University. They noted that in the SV-5/BHK-21 fusion system, the nuclei of giant cells became aligned in long parallel rows. By a combination of time lapse cinematography and electronmicroscopy they showed that the nuclei of these fused cells migrated along cytoplasmic

channels demarcated by microtubules. Treatment with colchicine, a microtubule poison, disrupted the characteristic architecture of these giant cells.

2. Nuclear activation. Harris and his associates (1966, 1967) showed that the nuclei of differentiated cells could undergo striking changes when these cells were fused with less differentiated cells. The nucleus of the chick red cell normally becomes condensed during the course of red cell differentiation and nucleic acid synthesis ceases (Cameron and Prescott, 1963). When a red cell is fused with a replicating HeLa cell the chick nucleus gradually swells and nucleic acid synthesis is resumed. This apparent reversal of differentiation has been called reactivation of the red cell nucleus and has been studied in considerable detail.

It turned out to be a general rule that somatic cells lacking DNA or RNA synthesis could be induced to do so by fusion with cells which did make the appropriate nucleic acid (Harris et al, 1966). In the case of the chick erythrocyte this was associated with a 20 - 30 fold swelling of the nucleus, and a 5 - 6-fold increase in dry mass (Harris, 1967; Bolund et al, 1969). The increase in nuclear volume correlated with the degree of stimulation of RNA synthesis, but the increase in dry mass was thought to be mainly due to the inflow of cytoplasmic proteins (Harris 1967). Quantitative spectrophotometric studies by Bolund and his coworkers (1967, 1968) showed that the red cell DNA was fully replicated and that it underwent striking changes in its structure. The DNA of the reactivated nucleus bound greater amounts of dyes like acridine orange or ethidium bromide and it was more susceptible to heat denaturation. It was thought that ionic changes in the nucleus could loosen up the structure of condensed chromatin, since similar changes were observed in unfused chick red

cells suspended in protein-free salt solutions (Ringertz and Bolund, 1969).

The ability of the reactivated chick nucleus to determine the synthesis of chick-specific gene products was demonstrated by Harris and his coworkers (1969) in a series of elegant experiments. The chick membrane antigens, introduced at fusion into mouse fibroblast x chick red cell heterokaryons, gradually disappear. These chick antigens, however, reappear in the heterokaryons after several days. This coincides with the appearance of a morphologic nucleolus in the reactivated chick nucleus. When embryonic chick red cells were used in these experiments both the chick antigens and nucleolus appeared somewhat earlier and in parallel. It was postulated that the nucleolus plays a part in the transport of chick messenger RNA into the heterokaryon cytoplasm. Destruction of the chick nucleolus by means of a u-v microbeam abolished the appearance of chick RNA in the cytoplasm and the reappearance of the membrane antigen. The synthesis of a different chick-specific gene product, inosinic acid pyrophosphorylase, a soluble cytoplasmic enzyme, was demonstrated in the same way (Harris and Cook; Cook 1970). It is interesting, however, that such chick red cell heterokaryons do not retain the ability to make hemoglobin (Harris, 1970). If less mature chick embryo erythrocytes, which do make hemoglobin, are fused with mouse tissue culture cells there is a transient stimulation of hemoglobin synthesis, followed by a gradual irreversible decrease.

3. <u>The regulation of the cell cycle in heterokaryons</u>. If the cells fused with Sendai virus are able to make DNA, their DNA synthesis usually becomes synchronized after fusion (Harris et al, 1966; Yamanaka and Okada, 1966; Johnson and Harris, 1969a). This implies that common cytoplasmic regulatory factors operate in such

multinucleate cells. An exception to this general finding was described by Johnson and Harris (1969b), who suggested that Ehrlich and HeLa cell nuclei could compete for such cytoplasmic factors in heterokaryons.

Rao and Johnson (1970a) have made further observations on the regulation of DNA synthesis and mitosis in multinucleate cells formed by fusing cells in different phases of the mitotic cycle. They showed that S nuclei rapidly induced DNA synthesis in G_1 nuclei, and, in turn were not inhibited by either G_1 or G_2 nuclei. In most cases entry into mitosis was also highly synchronized. Another striking effect could be achieved by fusing cells in mitosis with interphase cells (Johnson and Rao, 1970). The chromatin of the interphase nuclei was induced to condense prematurely and the nuclear membrane broke down. The pattern of condensation depends on the exact portion of the cycle which the interphase cell occupied at the time of fusion. The chromatin of G_1 and G_2 phase nuclei condensed to form chromosomes with single and double chromatids respectively. The chromatin of S phase nuclei condensed less completely and formed a patchwork of large and small fragments interspersed with non-condensed material. The condensation of S phase chromatin reduced, but did not abolish, DNA replication. The HeLa cell inducer of premature chromosome condensation is effective in a wide variety of cells including those of the mosquito (Johnson et al., 1970). It has been suggested that certain cations and polyamines play a role in this process (Rao and Johnson, 1970b).

Chromosome pulverization after cell fusion has been described by other workers and is probably a related phenomenon (Saksela et al., 1965; Sandberg et al., 1966; Ikeuchi and Sandberg, 1970).

4. The detection of latent viral genomes in transformed cells. Oncogenic viruses are able to transform many types of cell in tissue culture. Virus-specified T antigens may persist in such transformed cells, but the viral genome is latent and no virus progeny is produced in the case of many DNA oncogenic viruses. Upon fusion with cells which can support virus production, the latent viral genome can yield infectious virus (Barski, 1970). This phenomenon has been called virus rescue and has been observed in several laboratories (Gerber, 1966; Koprowski et al., 1967; Watkins and Dulbecco, 1967; Svoboda et al., 1967). Weiss and her coworkers (1968; Weiss, 1970) have shown that the SV-40 genome is probably incorporated into several of the human chromosomes in human x mouse hybrids.

The mechanism by which the virus genome becomes activated is not understood. It is not due to "simple" complementation between the 2 cells, since only a fraction (1/10 - 1/20) of heterokaryons produces virus, even though virtually all the non-producing cells contain at least one copy of the latent virus genome (Watkins and Dulbecco, 1967). If the transformed cells are treated with iododeoxyuridine or 8-azaguanine before fusion the proportion of heterokaryons which produces SV-40 virus is strikingly increased (Watkins, 1970). Moreover, it has been established that the transformed nucleus of such heterokaryons is the initial site of virus production (Wever et al., 1970). It is therefore clear that the state of the latent viral genome in the transformed nucleus is crucial in determining whether cell fusion will make the presence of virus detectable or not. The rescue phenomenon is clearly distinct from the observation by Enders and his associates (1967, 1968) that cells which normally resist poliovirus infection can replicate poliovirus which has been introduced into the cell by fusion with Sendai virus.

This summary of fused cell systems illustrates the imaginative way in which cell fusion has been used to study a variety of problems in cell biology.

D. The Properties of Hybrid Cells

Heterokaryons which make DNA usually enter mitosis (Harris et al., 1966). Many fused cells abort at this stage, for reasons which may include asynchronous chromosome condensation and difficulty in forming an appropriate spindle (Migeon and Childs, 1970). A proportion of hybrid cell colonies can, however, become established as vigorously growing cell lines over the course of a few weeks. These hybrids usually arise from the fusion of only two cells (Ruddle, 1970).

1. The isolation of hybrid cells depends on the availability of some procedure which selects for their growth, while eliminating the parental cells from the growing population. The HAT selection system of Littlefield (1964) has been widely used for this purpose. It depends on the availability of parental cells which lack either thymidine kinase (TK) or hypoxanthine-guanosine phosphoribosyl transferase (HGPRT). A variety of cell lines has been produced with the help of chemical mutagens and isolated on the basis of their ability to grow in bromodeoxyuridine (these cells therefore lack TK) or azaguanine (these cells lack HGPRT). If aminopterin is used to shut off endogenous purine and pyrimidine synthesis the TK^- or $HGPRT^-$ cells die because they cannot utilise exogenous thymidine or hypoxanthine present in HAT medium. Fused cells are enabled to grow by complementation of their enzyme deficiencies.

The use of non-dividing cells or cells which cannot adhere to the surface of the culture vessel can be exploited. In such a half-

selection system it is only necessary to kill one of the parent cells with HAT.

Other means of hybrid selection are also available. Puck and his coworkers (1970) have produced several variant cell lines which are auxotrophic for simple nutrients. Cells from parents with certain genetic defects, e. g. Lesch-Nyhan syndrome (Siniscalco, 1970), galactosemia (Nadler et al., 1970) or orotic aciduria (Krooth and Sell, 1970) express their enzyme deficiency in tissue culture and biochemical selection methods have been devised.

2. The chromosome changes which hybrid cells undergo as they evolve in tissue culture are important when it comes to using hybrid cells for genetic analysis. Intraspecific crosses often retain most of the original chromosomes contributed by the parent cells, although minor losses of chromosomes, usually less than about 10%, may occur (Migeon and Childs, 1970; Engel et al., 1969). These are called conservative hybrids. Hybrids produced by fusing mouse x man (Weiss and Green, 1967) or Chinese hamster x man (Kao and Puck, 1970) rapidly and preferentially lose the human chromosomes. Such "reduced" hybrids may lose all their human chromosomes except those necessary for the hybrid to survive in a particular selective medium (Weiss and Green, 1967). The mechanism by which chromosome reduction occurs is not understood, although asynchrony of chromosome condensation may be important, especially in those hybrids derived from parent cells which differ widely in their rate of growth (Migeon and Childs, 1970). It is possible that chromosome loss may occur in discontinuous steps, during which several chromosomes are eliminated at the same time, rather than in a continuous manner (Kao and Puck, 1970). Ruddle (1970) has separated hybrid clones very soon after fusion and shown that some of these clones do

have high numbers of human chromosomes which may then be retained in a stable fashion. Possibly the hybrids with fewer human chromosomes grow faster and would thus tend to predominate in uncloned populations.

3. Genetic analysis. Conservative hybrids have been useful in studying complementation between different alleles such as those occurring in genetic disorders like galactosemia (Nadler et al., 1970). Reduced hybrids have been used extensively for linkage analysis (Nabholz et al., 1969; Ruddle et al., 1970). A large number of man-mouse clones is isolated and screened for the presence of a battery of biochemical markers. There is virtually no limit to the number of suitable phenotypic markers, since most mouse and human proteins differ sufficiently for identification. Techniques like starch gel electrophoresis or isoelectric focusing can be used to detect differences in molecular charge, as well as hybrid molecules; human and mouse enzymes also often differ in thermal stability (Migeon and Childs, 1970; Ruddle, 1970). The loss of human chromosomes resembles segregation during meiosis and linkage groups can be assigned on the basis of persistent cosegregation. The presence of phenotypic markers can also be correlated with particular chromosomes in cases where human and mouse chromosomes can be distinguished. The chromosome which carries the gene for human thymidine kinase has been identified in reduced hybrids (Weiss and Green, 1967) and a possible linkage occurs between LDHB and peptidase B (Ruddle, 1970).

Ruddle (1970) has discussed some of the difficulties which are implicit in the above oversimplified scheme and which complicate the geneticist's task. It is not known for certain that the loss of human chromosomes is truly random. It is easy to isolate inde-

pendent clones, but these are not necessarily homogeneous. Secondary cloning from a mosaic population may select rare cells, so that a particular marker may apparently be absent and then reappear. Although chromosome rearrangement is not common it has been identified with some certainty in reduced hybrids which retain their human TK enzyme (Migeon et al., 1969), but no distinct human chromosome (Migeon and Miller, 1968). Chromosome rearrangement can also be exploited to map chromosomes. Miller and his coworkers (1971) showed that G6PD and HGPRT loci must be some distance apart on the human X chromosome since they segregate frequently in hybrids. Mouse chromosomes, which are typically acrocentric, are often difficult to distinguish from human chromosomes since centric fusion results in the presence of biarmed chromosomes in many mouse cell lines (Ruddle, 1970). Accurate measurement of arm length is also not definitive. In situ hybridization, using radioactive nucleic acids of very high specific activity, has recently been used to identify mouse satellite DNA near the centromere regions of mouse chromosomes (Ruddle et al., 1970) and even simpler cytochemical techniques may soon be available to facilitate the identification of individual chromosomes on morphologic grounds (Ruddle, personal communication).

4. Phenotype expression. The final assumption on which genetic analysis rests is that genes which are present are always fully expressed. This seems to be true for those gene products which are found in all cells, like many isozymes or H-2 antigens. In the case of cell-specific gene products there is considerable variation in gene expression. I have summarised some of the available information in Table I, according to whether a particular gene product was fully expressed, absent or considerably diminished, expressed at an

TABLE I

GENE EXPRESSION IN HYBRID CELLS

Type	Trait Studied	Hybrid	Ref
1A Codominance	Isozymes	Numerous Intra- and Interspecific	Ephrussi and Weiss, 1967; Boone and Ruddle, 1969; Nabholz et al., 1969
	H-2 antigens	mouse x mouse	Spencer et al., 1964; Klein et al., 1970; Silagi, 1967
		mouse x man*	Kano et al., 1969
	Species antigens	mouse x man	Weiss and Green, 1967; Nabholz et al., 1969
		various interspecific	Kano et al., 1969
	Electric excitability	mouse neuroblastoma x mouse fibroblast	Minna et al., 1971
B Complementation	Galactosemia	various human diploid	Nadler et al., 1970
	Nutritional auxotrophs	various Chinese hamster	Kao et al., 1969
	G-6-PD, HGPRT (X linked)	human diploid x human diploid	Siniscalco et al., 1969
		human diploid x human heteroploid	Silagi et al., 1969
2A Absence or considerable Reduction (No reversion)	Pigment	hamster melanoma x mouse fibroblast	Davidson et al., 1966, 68
		mouse melanoma x mouse fibroblast	Silagi, 1967
	Growth hormone	rat pituitary tumor x mouse	Sonnenschein et al., 1968
	Teratoma morphology	mouse teratoma x mouse	Finch and Ephrussi, 1967
	28s RNA (human)	mouse x man	Eliceiri and Green, 1969
	Immunoglobulins or chains	mouse myeloma x mouse fibroblast	Periman, 1970; Mohit and Fan, 1970
	S-100 Glyceraldehyde-3-P dehydrogenase, basal level - inducibility	rat glial(†) x mouse fibroblast	Davidson and Benda, 1970
	Tyrosine amino transferase, basal level - inducibility	rat liver tumor(†) x mouse fibroblast	Schneider and Weiss, 1971
B Reduction with occasional reversion in subclones (associated with chromosome loss)	Es-2	mouse tumor(†) x human fibroblast	Klebe et al., 1970
	H-2 antigens	Ehrlich ascites x mouse fibroblast(†)	Klein et al., 1970
3. Intermediate	Collagen Hyaluronic acid	mouse fibroblast x mouse fibroblast	Green et al., 1966
	Folate reductase	hamster (high) x hamster (low)	Littlefield, 1969
	Glyceraldehyde-3-P Dehydrogenase	rat glial(†) x mouse fibroblast	Davidson and Benda, 1970
	OMP decarboxylase	human orotic aciduria (low) x human (high)	Silagi et al., 1969
4. Increased or "Induced"	Interferon production Interferon sensitivity	Syrian hamster(†) x mouse	Carver et al., 1968
	Hyaluronic acid	mouse x Chinese hamster lung cell lines	Koyama and Ono, 1970

*Reduced hybrids, some clones lost human marker, probably because of chromosome loss.

(†)Parent which has particular marker.

intermediate level, or even increased, in the particular hybrid cells studied. This diversity, no doubt, reflects the variety of regulatory effects, as yet undefined, which may operate at any step in the path from gene to cell product.

In some instances (see Es-2 and H-2 in Table I) markers which were not expressed in hybrid cells reappeared as more chromosomes were lost. This behavior has been used as an argument for regulator genes and chromosomes, but in both cases one cannot exclude selection artifacts during cloning (Es-2) or during in vivo growth (H-2).

An early goal of somatic cell hybridisation was to perform a genetic analysis of malignancy (Barski, 1970). Initial studies suggested that hybrids formed between cell lines of high and low malignancy remained highly malignant (Barski, 1970; Scaletta and Ephrussi, 1965; Silagi, 1967; Defendi et al., 1967). This area has become the centre of considerable controversy since Harris and his coworkers (1969), in subsequent studies, claimed to suppress malignant cell behavior by fusion with non-malignant cells. They also described a reversion to malignancy in "segregants", which had fewer chromosomes. The surface properties examined in these hybrid cells, e.g. H-2 antigens, did not correlate with their malignancy (Klein et al., 1970). Much work, however, remains to be done with hybrid cells before the relationship between chromosomes, surface properties and cell malignancy is understood.

* * * * * *

It is necessary to review selected aspects of DNA synthesis in animal cells and to summarise our current knowledge of macrophage plasma membrane receptors, as background for the experimental studies to be reported.

E. The Control of DNA Synthesis in Animal Cells

Our knowledge of the biochemistry of DNA synthesis in animal cells is fragmentary and can be briefly summarised as follows. It is likely that DNA replication proceeds by a semiconservative mechanism (Taylor, 1969). The replication process is clearly ordered, but considerable variation exists with respect to its timing within the same or different chromosomes (Prescott, 1970). Initiation of DNA synthesis probably occurs at multiple sites (Huberman and Riggs, 1968) and recent evidence suggests that small fragments of newly made DNA are joined together to form larger units (Sakabi and Okazaki, 1966; Schandl and Taylor, 1969). Surprisingly little is known about the enzymes which play a role in DNA synthesis. Among these enzymes one can include a DNA polymerase, a ligase and various enzymes which could generate the deoxyribonucleoside triphosphates. Perhaps there are enzymes to separate the strands of coiled DNA and endonucleases may help to generate new starting points. There is some indirect evidence, and much speculation, that specific initiator proteins (Kuempel, 1970) exist in animal cells (Prescott, 1970) and even less evidence that a primary membrane attachment site is important for DNA replication (Comings and Kakefuda, 1968).

We are on firmer ground, at a descriptive level, in discussing the relationship between DNA synthesis and the life cycle of animal cells. Based on observations that the period of DNA synthesis is limited to a part of the cell cycle, 4 stages are defined (Howard and Pelc, 1953): mitosis (M); a period before DNA synthesis (G_1); DNA synthesis itself (S) and a period before mitosis (G_2). The length of each period is characteristic for a cell-type, although there is considerable variation from cell to cell, most often in the duration of

G_1 (Prescott, 1970). Cells such as the hepatocyte have a very long G_1 period, called G_o (Quastler and Sherman, 1959; Lajtha, 1963), during which they remain dormant, but are apparently able to enter S if triggered by partial hepatectomy. Other cells, like the mature chick erythrocyte, leave the cell division cycle permanently during the course of their differentiation (Cameron and Prescott, 1963).

It is thought that the life cycle of each cell involves the orderly expression of specific genetic information (Baserga, 1968; Mueller, 1969). This assumption is based mainly on studies of synchronised cell populations which suggest that RNA and protein synthesis may be required for progress through different phases of the cell cycle. The mechanism by which cells remain dormant in G_o or reenter S, as in a continuously proliferating population, is not known. It is widely held to be the most important control step in cell multiplication since only a few types of cell naturally come to rest in G_2 (Gelfant, 1966) and the induction of DNA replication usually results in mitosis (Baserga, 1968).

F. Macrophage Plasma Membrane Receptors

1. Introduction. In 1960 Boyden and Sorkin showed that rabbit spleen cell suspensions, incubated with antisera and then washed, were able to bind antigens like ^{131}I-labeled human serum albumin. Globulin components of the antiserum had become attached to some of the spleen cells in such a way that they were subsequently capable of specifically adsorbing antigen (Boyden, 1963). These antibodies were called cytophilic (Boyden, 1963; Sorkin, 1963) and could also react with pure populations of macrophages (Jonas et al., 1965; Berken and Benacerraf, 1966). The macrophage plasma membrane is thought to carry distinct receptor molecules which react apeci-

fically with certain classes of antibody, either free or in the form of an antigen-antibody complex (Uhr, 1965). Studies with a variety of cells, sera and antigens have shown that human blood monocytes carry similar receptors (LoBuglio et al., 1967; Huber and Fudenberg, 1968) and that 7S and 19S antibodies, complement components or α_1 globulins could all mediate binding (Nelson, 1968).

2. Cell specificity. Studies by Lay and Nussenzweig (1969a, b) have delineated the following different receptors for immunoglobulins and complement on mouse leukocytes (Table II). These differ with regard to cell distribution, susceptibility to trypsin treatment and requirement for divalent cations. Perhaps the best studied, and least controversial, are the receptors for 7S immunoglobulins. Such γG receptors have hitherto only been found on specialised phagocytic cells like the peritoneal macrophage and blood monocyte. Tissue macrophages, e.g. from the lung or spleen, also carry these receptors, but other cell types, like peritoneal fibroblasts, do not (Jonas, 1965; Nelson, 1969). In fact, antibody coating stimulates particle uptake in the macrophage, but depresses uptake in L cell fibroblasts (Rabinovitch, 1969). In most studies the bulk of lymphoid cells do not display γG receptor activity (Lay and Nussenzweig, 1969a, b). Polymorphonuclear (PMN) cells vary in reactivity. Messner and Jellinek (1970) showed that many Rh antibodies failed to interact with PMN, while other hyperimmune sera did. A recent report states that IgE, a different class of antibody, is selectively cytophilic for basophils (Ishizaka et al., 1970).

3. Nature of antibody and binding reaction. Rabbit cytophilic antibodies often fall in the 7S class (Nelson, 1969); in the guinea pig the reactive species are the slower migrating γ_2 antibodies, which fix complement (Berken and Benacerraf, 1966). Heterologous as

TABLE II

Receptors for IgG and Complement Factors on Mouse Leukocytes

Receptor	Cells Detected	Effect of Trypsin Treatment	Dependence on divalent cations
7S Ig	Peritoneal macrophage Polymorphs Blood monocytes	Nil	Not
19S IG	Peritoneal macrophages	Nil	Depends on Ca^{++}
Complement (A)	Lymph node and thoracic duct lymphocytes Not thymus lymphocytes	Destroyed	Not
Complement (B)	Peritoneal macrophages Polymorphs Blood monocytes	Destroyed	Depends on Mg^{++}

(Taken from W. Lay and V. Nussenzweig, 1969b)

well as isologous antisera have been used to study binding, although intraspecific combinations of white cell and antiserum may be more efficient (Berken and Benacerraf, 1966; Abramson et al., 1970a). Several groups have demonstrated γG receptors on human blood monocytes by using red cells coated with noncomplement fixing Rh antibodies, or with various agglutinins (Huber et al., 1968, 1969; Abramson et al., 1970a, b). Purified myeloma proteins which belong to IgG subclasses 1 and 3, but not 2 or 4, bind most effectively to monocytes (Huber et al., 1968; Abramson et al., 1970b; Inchley et al., 1970). Myeloma proteins were used to coat red cells for direct binding to monocytes and were also able to inhibit one another in competition experiments (Abramson et al., 1970b; Huber et al., 1971). Since myeloma proteins also inhibited binding in a heterologous system (Huber et al., 1971) it is likely that human monocyte and animal macrophage receptors are similar in structure.

Antibody binds to macrophages at $4^{o}C$ as well as $37^{o}C$, and binding is independent of divalent cations (Berken and Benacerraf, 1966). When antigen-antibody complexes are used, binding to the macrophage can be detected at much lower concentrations of antibody. It is not clear whether this is due to a conformational change in the antibody, after combination with a specific antigen, or due to stabilization of the binding reaction by multivalent antigens (Phillips-Quagliata et al., 1969). The antibody can be coupled to the red cell by a non-immunological method, e. g. with $CrCl_3$ (Abramson et al., 1970a), but data are not available to compare the sensitivity of the immune and non-immunologic detection systems. Relatively low concentrations of "non-specific" IgG compete very effectively for binding with antigen-antibody complexes (Berken and Benacerraf, 1966). Berken and Benacerraf (1966) also found that cytophilic antibody elutes readily from the macrophage at $37^{o}C$.

These observations suggest that a dynamic equilibrium exists between bound and free IgG in such a reaction system. When the red cells are coated with specific antibody the binding reaction is less reversible. Characteristic rosettes are formed as the antibody coated red cells bind to macrophages. Ingestion of the antibody coated red cells is then triggered, especially in the presence of serum and at 37°C (Rabinovitch, 1968). Electron microscopy has shown that the gap between the membranes of the red cell and monocytes is preserved, although some "adherent" regions are found in which the gap is obliterated (LoBuglio et al., 1967; Abramson et al., 1970a). The monocyte then envelopes the red cell, which may become spherocytic during the reaction.

The macrophage receptor binds through the Fc portion of the antibody molecule (Berken and Benacerraf, 1966; Uhr and Phillips, 1966; Abramson et al., 1970b, Inchley et al., 1970). Pepsin destroys the Fc region and can abolish binding. The Fab and Fc fragments, isolated after papain treatment at neutral pH, have been tested individually; the Fc fragment can bind efficiently and block binding by the whole antibody molecule whereas the Fab fragment is inactive.

Several other biologic properties of antibody molecules reside in the Fc fragment, e. g. the ability to fix complement, attach to skin, and to cross the placenta or gut (Cohen and Porter, 1964). The Fc portion also carries some of the Gm allotypic antigens (Cohen and Milstein, 1967) and is able to combine selectively with staphylococcal A protein (Forgren and Sjoquist, 1966; Kronvall and Williams, R. C. Jr., 1969). The Fc piece of rabbit γG globulins has a molecular weight of about 48,000, and is relatively homogeneous, confirmed by its tendency to crystallize. The different subclasses show some differences in primary sequence (Frangione et al., 1964), but it is

not known, which, if any, of these are responsible for the rather striking differences in biologic function. Attempts have been made to obtain smaller fragments of the Fc piece which are biologically active. By further proteolytic digestion, especially under acid conditions, it is possible to obtain rather large C terminal fragments, but these invariably lack biologic activity (Frommel and Hong, 1970). An N-terminal fragment, isolated after cyanogen bromide cleavage, binds complement (Kehoe and Fougereau, 1969). In contrast, reduction of disulfides has been shown to destroy the ability of the Fc fragment to bind to the monocyte receptor (Abramson et al., 1970b). It is possible that the conformation of the Fc fragment is essential for much of its biologic activity. It is interesting that the Fc piece carries most of the carbohydrate moiety of the YG molecule (Cohen and Milstein, 1967), but very little is known about its chemistry or function. Inchley and his coworkers (1970) found that the total carbohydrate content, a rather crude measure, does not correlate with the ability to bind to the macrophage.

4. Nature of cell receptor. Nothing is known about the chemistry of the macrophage Fc receptor. This receptor is strikingly resistant to treatment with trypsin or other proteolytic enzymes (Howard and Benacerraf, 1968; Davey and Asherson, 1967; LoBuglio and Rinehart, 1970); the receptors for complement and α_1 globulins are trypsin sensitive (Nelson, 1968). This, of course, does not mean that the Fc receptor does not contain protein. In some cases antibody which is known to be adsorbed to a cell cannot be released by proteolytic treatment (Kossard and Nelson, 1968); this suggests that the surface topography of the cell may limit enzyme access. The Fc receptor also resists treatment with neuraminidase. Various crude phospholipases have been used to destroy the receptor, but it is

likely that extensive cell damage also occurred in these experiments (Allen and Cook, 1970). Sulfhydryl and carbohydrate groups may play a role in receptor activity, on the basis of treatment with sulfhydryl reactive agents and periodate (Howard and Benacerraf, 1968; Davey and Asherson, 1967; LoBuglio and Rinehart, 1970). These experiments have not been adequately controlled for non-specific cell damage nor have the postulated chemical reactions been demonstrated. Metabolic inhibitors like fluoride or dinitrophenol apparently do not affect the receptor. This catalogue of our ignorance concludes with the observation that nothing is known about the distribution of the Fc receptor on the macrophage surface, its occurrence in other membranes of the cell, or its synthesis and turnover.

Even the biologic function in vivo is mysterious. There is no doubt that antibody coated infectious agents can be rapidly ingested in vitro, but the high concentration of nonspecific IgG in tissue fluids could compete effectively for attachment (Berken and Benacerraf, 1966). Other speculative functions include a role in red cell destruction (LoBuglio et al., 1967) and tumor rejection, the latter based mainly on in vitro studies by Granger and Weiser (1966).

G. Introduction to the Thesis Problem

We used cell fusion to study two important general problems in cell biology, the control of DNA synthesis initiation and the expression of membrane receptors. By fusing two cell types which differ strikingly with respect to both these properties, we could pose the question: which cell will predominate if we combine them? In this way we hoped to gain insight into control mechanisms in animal cells.

Mouse peritoneal macrophages and mouse melanoma cells were chosen for the present fusion experiments. They differ in DNA

synthesis, membrane receptors, and fuse readily with each other upon treatment with Sendai virus. The mouse peritoneal macrophage does not usually make DNA during in vitro cultivation (v. Furth and Cohn, 1968). This cell derives from the bone marrow and enters the peritoneal cavity through the blood stream. During the course of its differentiation the macrophage stops making DNA. The macrophage can be considered a true G_o cell since it may be stimulated to make DNA again under certain circumstances. The most convincing evidence that mature macrophages can re-enter S comes from the study by Virolainen and Defendi (1967), who showed that "conditioned medium", obtained from other replicating mouse cells, contained a factor(s) which induced DNA synthesis in macrophages cultivated in vitro for some days. Such macrophages can also be stimulated to make DNA by infection with oncogenic viruses like SV-40 or polyoma (Mallucci, 1969; Mauel and Defendi, 1968). Several studies suggest that macrophage replication can be triggered in vivo as well, but it is often not clear whether these macrophages are truly mature, before stimulation, or whether less mature macrophages are involved (Khoo and Mackannes, 1964; Forbes, 1965).

By fusing mature macrophages with rapidly growing melanoma cells we hoped to gain insight into the nature of the G_o block in macrophage DNA synthesis.

The Fc receptor is an excellent marker for use in cell fusion systems. All macrophages continue to express this receptor in tissue culture and it is remarkably stable to treatment with a variety of enzymes and drugs. It is simple to assay in individual cells. Moreover, since it is unique to the macrophage, it may be considered a "differentiation" antigen (Boyse and Old, 1969). By fusing macrophages with melanoma cells which lack these receptors one could not

only trace the fate of the macrophage membrane in the heterokaryons, but also hope to learn about the expression of cell-specific traits in animal cells.

Another membrane marker was also available in this fusion system. The plasma membrane of the macrophage is a rich source of adenosine triphosphatase (ATPase) activity (North, 1966). This enzyme requires only divalent cations for full activity. Its function is obscure, but ATPase activity has been implicated in phagocytosis and macrophage spreading (North, 1968;1969). ATPase activity can be detected in living cells by biochemical assay, as well as by cytochemical procedures on fixed cells. The melanoma cells have so much less ATPase activity than the macrophages that it was possible to distinguish between the two types of cell membrane on this basis.

The macrophage has other properties which make it a useful partner in cell fusion experiments. It is easy to cultivate pure populations in vitro and the macrophage is able to synthesize significant amounts of lysosomal enzymes, e. g. acid phosphatase, providing another useful cytochemical marker (Cohn and Benson, 1965).

The melanoma cells used in these experiments were not pigmented, although pigment can form after treatment with cytosine arabinoside or cultivation under agar (Silagi, 1969).

CHAPTER II

THE PREPARATION AND MORPHOLOGIC PROPERTIES OF MACROPHAGE-MELANOMA CELL HETEROKARYONS

Materials and Methods

Media. Macrophage culture medium consisted of medium 199 (Microbiological Associates) and 10 or 20% newborn calf serum (Grand Island Biological) which had been heated for 30 minutes at 56°C and passed through a millipore filter, pore size 0.45μ. Penicillin G (Lilly) was added at 20 units per ml. The complete medium will be referred to as 199M.

Melanoma cell culture medium consisted of Dulbecco's medium supplemented with 10% calf serum and 10% tryptose phosphate broth and will be referred to as DM.

Solutions. PBS refers to phosphate buffered saline (Dulbecco and Vogt, 1954) and PD to PBS without Ca^{++} and Mg^{++}. TV consisted of 0.25% trypsin (Nutritional Biochemical Corp.) and 0.05% versene in PD. Glutaraldehyde (Fisher Scientific Co.) was used as a fixative at a concentration of 1.25% in PD.

Cells. Cells were obtained as follows: Melanoma cells, non-pigmented clone NPN87, isolated by Dr. S. Silagi of The New York Hospital from line B16 cells; baby hamster kidney cells (BHK21-F) from Dr. K. V. Holmes, The Rockefeller University; HeLa, L2 and L929 cells from Dr. L. Sturman, The Rockefeller University.

Method of Cell Culture. Macrophage cultures were set up according to the method of Cohn and Benson (1965), with some modifications.

Female mice from the NCS colony maintained at The Rockefeller University and weighing 25 to 30 grams were used for all studies. Peritoneal cells were collected in PD containing 20 units of heparin per ml and resuspended in 199 M after centrifugation.

The cell suspension was pipetted in 0.05 ml amounts onto 12 mm coverslips which had been placed in tissue culture dishes (Falcon Plastics). Each mouse yielded about 2×10^6 cells from which 10 coverslips could be prepared. After one hour's incubation at 37°C and in the presence of 5% CO_2, nonadherent cells were removed by two vigorous washes with 199. The cells were then further incubated in 199 M.

Melanoma Cell Culture. Cultures were grown in DM, harvested with TV and one per cent of the cells replated weekly. Cells for fusion experiments were routinely cultivated overnight to provide a standard, exponentially growing population.

Microscopy. Coverslip preparations were fixed in glutaraldehyde for 10 minutes at 4°C and examined by oil immersion phase contrast microscopy using a Zeiss Ultraphot, Model II. Photographs were taken using 4" x 5" Panatomic X film.

On occasion preparations were fixed in methanol and stained by the Giemsa May-Grünwald method.

Preparation of Sendai Virus. Sendai virus was originally obtained from Dr. P. Choppin of The Rockefeller University. Stocks of virus were grown in hen's eggs, partially purified and stored, following the method of Harris and coworkers (1966).

Hemagglutination titers were determined using serial dilutions of virus in PBS containing 0.5% BSA (Choppin et al., 1958). A 0.5% suspension of chick red blood cells was incubated with virus for 70

minutes at 4°C.

Viral infectivity was assayed in 10 day fertile hen's eggs. Ten-fold dilutions of virus were injected into eggs and the hemagglutination titer of the allantoic fluid measured after 2 days' incubation at 35°C. The EID 50 was calculated using the Reed-Muench formula (1938).

Ultraviolet irradiation was used to inactivate virus. Concentrated virus was thawed immediately before use and irradiated with a Sylvania germicidal lamp (G15T8) for 9 minutes at a distance of 15 cm. Irradiated virus was diluted in 199 M to the desired final concentration and kept in an ice bath until used.

Virus specific hemadsorption was studied by the method of Harris and coworkers (1966).

Treatment with unirradiated Sendai virus leads to the production of viral hemagglutinin in macrophages as well as in melanoma cells, although this process takes longer in the macrophage. As a result of ultraviolet irradiation the viral infectivity titre fell from 8.5 to 0.5 log units, as measured in hen's eggs, without significant reduction of fusion activity. Irradiation of virus effectively abolished its ability to produce viral hemadsorption in macrophages as well as in melanoma cells.

Cell Fusion Technique. a) Macrophage homokaryons. Macrophages were cultivated for one day or longer at a cell density of 2×10^5 cells per cm^2. Coverslips were washed twice with 199, drained and placed in new tissue culture dishes (Falcon Plastics, 60 or 35 mm in diameter). A viral suspension containing 500 to 2000 hemagglutinating units of virus in 0.05 ml was carefully added to each coverslip. After one hour's incubation at 37°C, the coverslips were washed twice with 199 and further incubated in 199 M.

In the procedure described above macrophages were cultivated on a coverslip for a day before viral treatment. Preliminary studies had shown that when a suspension of peritoneal cells was treated with virus the cells did not attach readily to glass and the recovery of fused cells was poor. After prior cultivation the yield of giant cells was improved dramatically and up to 50% of all macrophages could be fused. Moreover, this method yielded a population of macrophages free of other cells originally present in the peritoneal washings.

b) Macrophage-melanoma cell heterokaryons. The melanoma cells were chosen as the fusion partner for macrophages after preliminary experiments with several lines, including HeLa, L2, L929 and BHK 21-F. Melanoma cells fuse more readily with macrophages and by choosing appropriate cell ratios it was possible to obtain good yields of 1:1 heterokaryons routinely.

A suspension of melanoma cells was obtained by washing a monolayer of cells twice with PD, followed by TV treatment for 3 minutes at 37°C. Two volumes of 199 M were added and the cells collected by centrifugation. The cells were resuspended in fresh 199 M and counted. Ten to twenty thousand melanoma cells were added to the macrophage monolayer on each coverslip and allowed to settle. After one hour of incubation the coverslips were washed and either treated immediately with virus or cultivated in 199 M for some hours before fusion.

c) Isolation of heterokaryons by means of trypsinization. For certain studies large numbers of 1:1 heterokaryons were required which were free of melanoma cells. A trypsinization procedure was devised which yielded about 1×10^5 heterokaryons, mainly 1:1 in type. This procedure was based on the finding that treatment with trypsin affects macrophages and melanoma cells differently. Whereas melanoma

cells round up and are readily detached from glass, macrophages spread and resist detachment. Early heterokaryons which contain macrophage membrane can be detached by more prolonged trypsinization. They subsequently reattach and spread out more rapidly than melanoma cells.

Macrophages (about 5 x 10^6 cells) were cultivated in 15 cm^2 T flasks for 1 to 3 days. One million melanoma cells were resuspended in 2 ml of 199 M and allowed to become attached to the macrophage monolayer for 1 hour at 37°C. After rinsing the cells twice with warm 199, 2 ml of 199 M, containing 5,000 hemagglutinating units of inactivated virus, were added to the monolayer and the culture kept at 37°C for 1 hour. Two to 4 hours after the addition of fresh medium the preparation was washed twice with PD and incubated for 5 to 10 minutes in TV solution at 37°C. When almost all the heterokaryons had been detached, the trypsin treatment was stopped by resuspending the cells in two volumes of 199 M. The cells were then plated onto coverslips at the desired cell density. After the heterokaryons had spread out, the non-attached cells, mainly melanoma cells, were washed away and, if necessary, removed by further mild trypsinization. Fresh medium was added and the purified population of heterokaryons cultivated in vitro at 37°C.

d) Dextran sulfate laden macrophages. On occasion dextran sulfate was used as a macrophage marker. Dextran sulfate 2 x 10^6 m. w., was obtained from Pharmacia. Macrophages were pretreated with dextran sulfate, a substance which is taken up and concentrated in discrete cytoplasmic vacuoles. The dextran sulfate is indigestible, nontoxic and stains metachromatically.

Macrophages were cultivated for one day and then exposed to 10 µg/ml dextran sulfate in 199 M for 1 to 2 days. Free dextran sul-

fate was removed prior to the addition of melanoma cells. After the melanoma cells had become attached to the monolayer, the preparation was washed again and treated with virus. Preparations were fixed with glutaraldehyde and stained with toluidine blue for examination.

Control experiments were performed identically except that the viral treatment was omitted. The dextran sulfate was never found in melanoma cells whereas every macrophage was heavily laden. Such macrophages showed no obvious ill effects when observed for 10 days and could phagocytose red cells as readily as untreated cells. Moreover, the macrophages exhibited increased spreading after dextran sulfate treatment so that fusion was facilitated.

Time Lapse Cinematography was used to study the formation of macrophage homokaryons with the help of Professor James Hirsch. Macrophage monolayers were cultivated for 2 - 4 days on 22 mm^2 coverslips. The coverslips were placed in perfusion chambers and treated with prewarmed Sendai virus. The fusion process was observed by oil-immersion phase contrast microscopy, using a 40 X objective and photographed on Kodak Plus-X Reversal film at 30 frames per minute.

Acid Phosphatase. Glutaraldehyde fixed preparations of heterokaryons were examined for the presence of acid phosphatase using the histochemical procedure of Cohn and Benson (1965). Twenty-five cells of each type were scored at different times.

Results

The treatment of cocultivated macrophages and melanoma cells with Sendai virus resulted in the efficient fusion of single melanoma cells with 1, 2 or 3 macrophages, as well as the fusion of from 2 - 5

macrophages. The heterokaryons were named 1:1, 2:1 or 3:1, depending on the number of macrophage nuclei. The ratio of heterokaryons of each class obtained in a typical experiment was 1:1 85%, 2:1 13% and 3:1 2%.

The Morphology of Fused Cells. Some of the morphological events associated with cell fusion are illustrated in Figs. 1 - 3. Homokaryons, as well as heterokaryons, underwent a striking reorganization after fusion. At an early stage in the formation of a giant cell the nuclei and lipid droplets were distributed at random and no distinct centrosphere region was apparent. After 1 to 5 hours, the nuclei became arranged in a circle about a common centrosphere. Lipid droplets were then found only outside this circle of nuclei. The treatment of cells with 10 μg/ml of colcemid disrupted the organization of giant cells (Fig. 3f). Colcemid treated giant cells lost their well-defined centrosphere region and the nuclei and lipid droplets were found randomly distributed throughout the cell.

In heterokaryons macrophage and melanoma nuclei were easily distinguished. Melanoma nuclei are larger, oval shaped and contain several prominent, bar-like, nucleoli. Macrophage nuclei are smaller, often bilobed and have unobtrusive "nucleoli". Within an hour of fusion the macrophage nuclei in heterokaryons started to swell and their nucleoli became more prominent. These changes, which became more striking with time, are illustrated in Figs. 3b and 3d.

The nuclei of unfused macrophages show a characteristic rim of heterochromatin when examined by electron microscopy. After swelling within a heterokaryon the chromatin structure appears more diffuse, like the euchromatin typically seen in the melanoma cell nucleus (Fig. 4).

Figure 1 (a-c)

Early stages in the fusion of mouse macrophages after the addition of Sendai virus. 1. 25% Glutaraldehyde fixation. Phase contrast x 2000.

Fig. 1a. Ten minutes after the addition of virus a narrow bridge has formed between two adjacent cells.

Fig. 1b. Twenty minutes after the addition of virus, the bridges are wider and contain refractile lipid droplets.

Fig. 1c. Thirty minutes after the addition of virus the fusion of three macrophages has resulted in the formation of a trinucleate homokaryon.

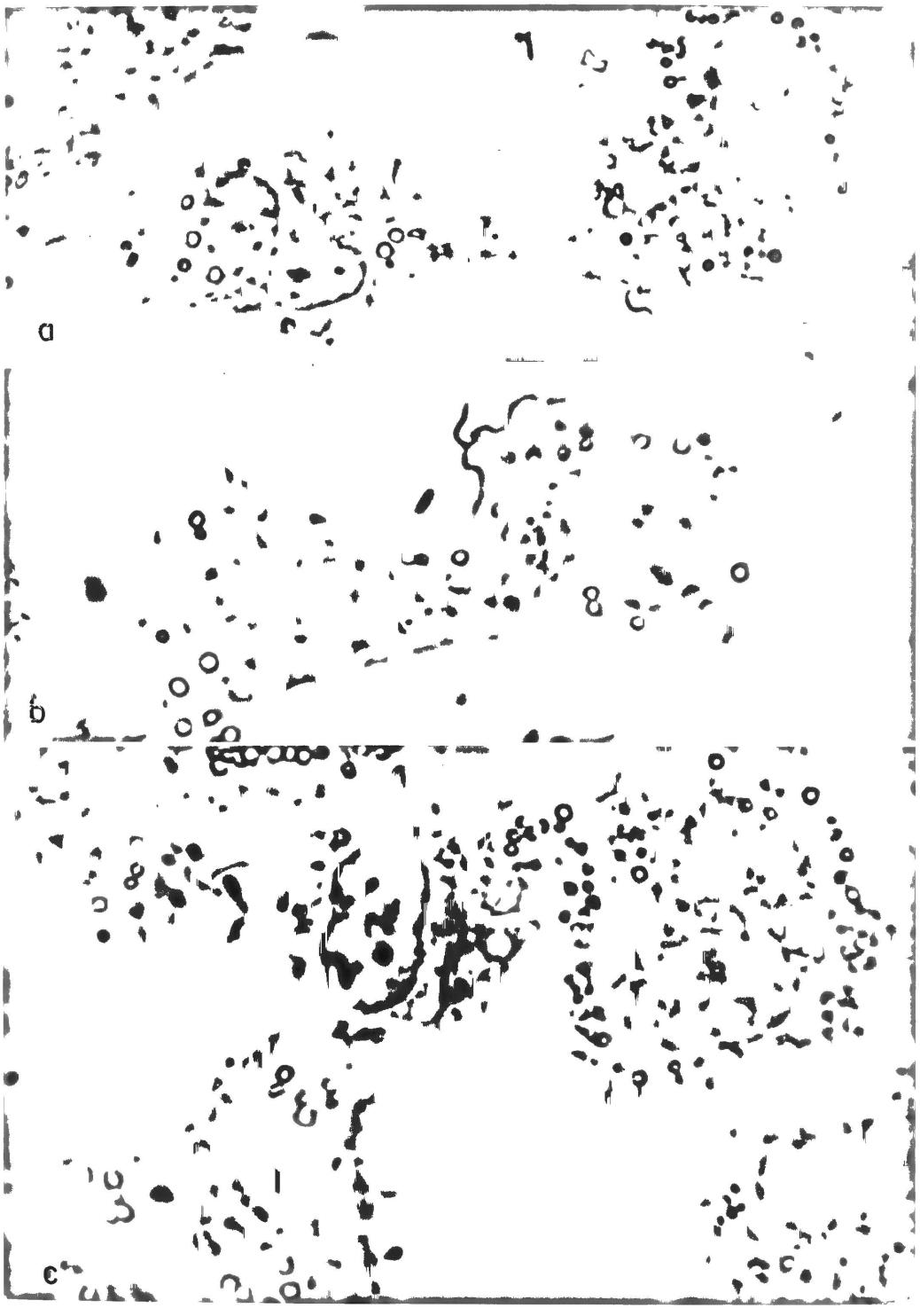

a
b
c

Figure 2 (a-f)

Later stages in the formation of macrophage homokaryons and macrophage-melanoma cell heterokaryons.

Fig. 2a. A trinucleate macrophage homokaryon two hours after the start of fusion. The cytoplasm is still unorganized and distinct clusters of lipid droplets remain about each nucleus. Phase contrast x 1200.

Fig. 2b. A macrophage homokaryon containing randomly distributed nuclei. May-Grünwald Giemsa stain x 1000.

Fig. 2c. A reorganized macrophage homokaryon. The three nuclei are arranged about a common centrosphere region and are rimmed with lipid droplets. Phase lucent pinocytic vesicles are present and the mitochondria are radially oriented. Phase contrast x 1000.

Fig. 2d. A macrophage homokaryon exhibiting nuclear reorganization. Stained x 1000.

Fig. 2e. An early stage in the fusion of two macrophages with a melanoma cell. Phase contrast x 1000.

Fig. 2f. A heterokaryon prior to the reorganization of cellular architecture. Stained x 1000.

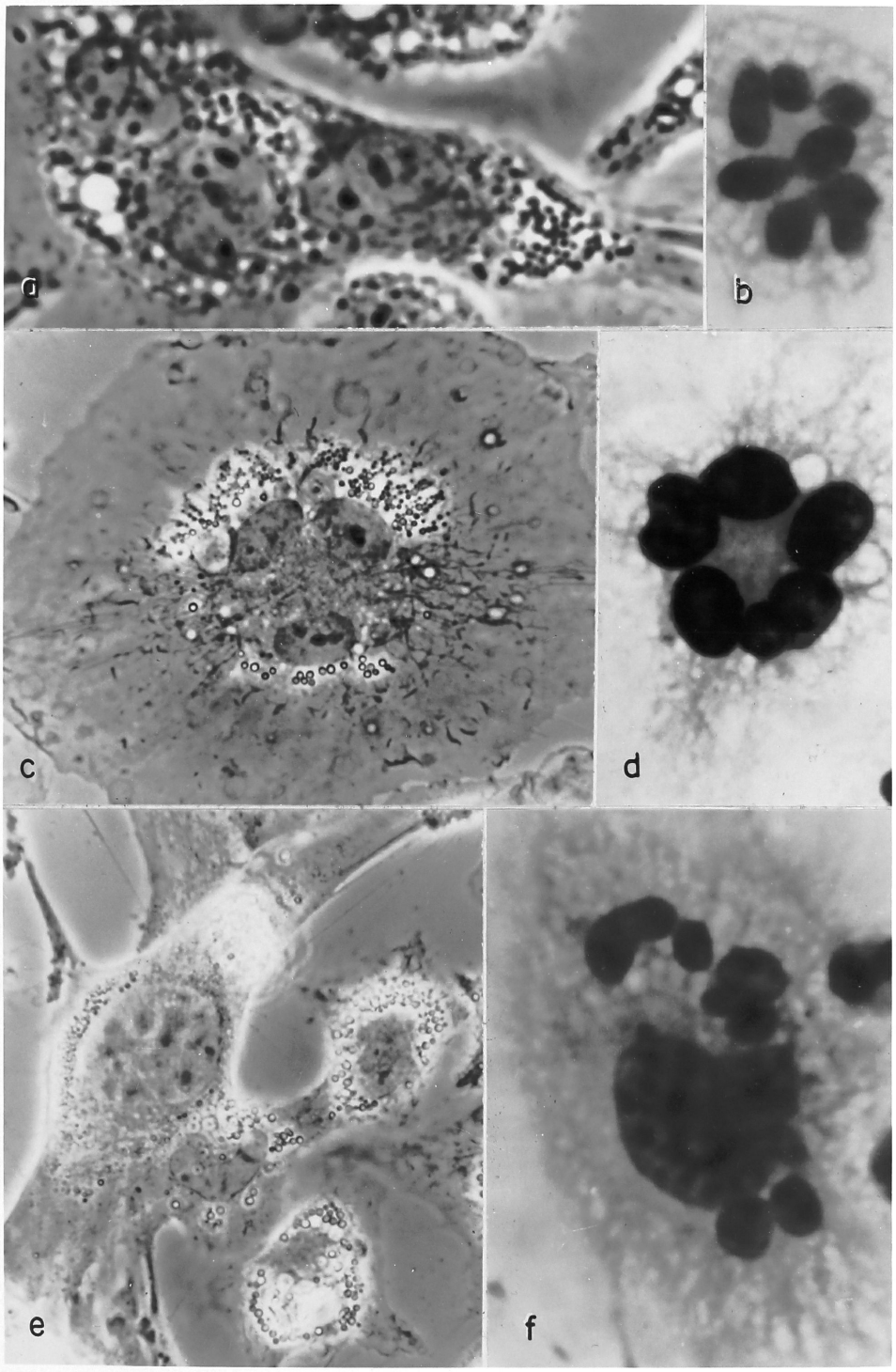

a
b
c
d
e
f

Figure 3 (a-f)

Examples of fused cells after in vitro cultivation.

Fig. 3a. A 1:1 heterokaryon after reorganization. The lipid droplets are arranged peripherally and the larger melanoma nucleus with its prominent nucleoli is evident. Phase contrast x 900.

Fig. 3b. A 1:1 heterokaryon two days after fusion. The macrophage nucleus on the right is now enlarged and contains a prominent nucleolus. Phase contrast x 1000.

Fig. 3c. A 3:1 heterokaryon after 24 hours of cultivation. The nuclei are now oriented and the three macrophage nuclei are larger than those of adjacent, unfused macrophages. Stained x 1000.

Fig. 3d. A 2:1 heterokaryon one day after fusion. The macrophage nuclei are enlarged and have a single prominent nucleolus. Phase contrast x 1000.

Fig. 3e. A large heterokaryon containing one melanoma nucleus and many macrophage nuclei. Striking reorganization about a common centrosphere region has occurred. Phase contrast x 900.

Fig. 3f. A macrophage homokaryon after five hours' exposure to colcemid (10 μg/ml). Lipid droplets and other organelles are randomly distributed. The nuclei (arrows) are located in the cell periphery in stubby pseudopods. Phase contrast x 1000.

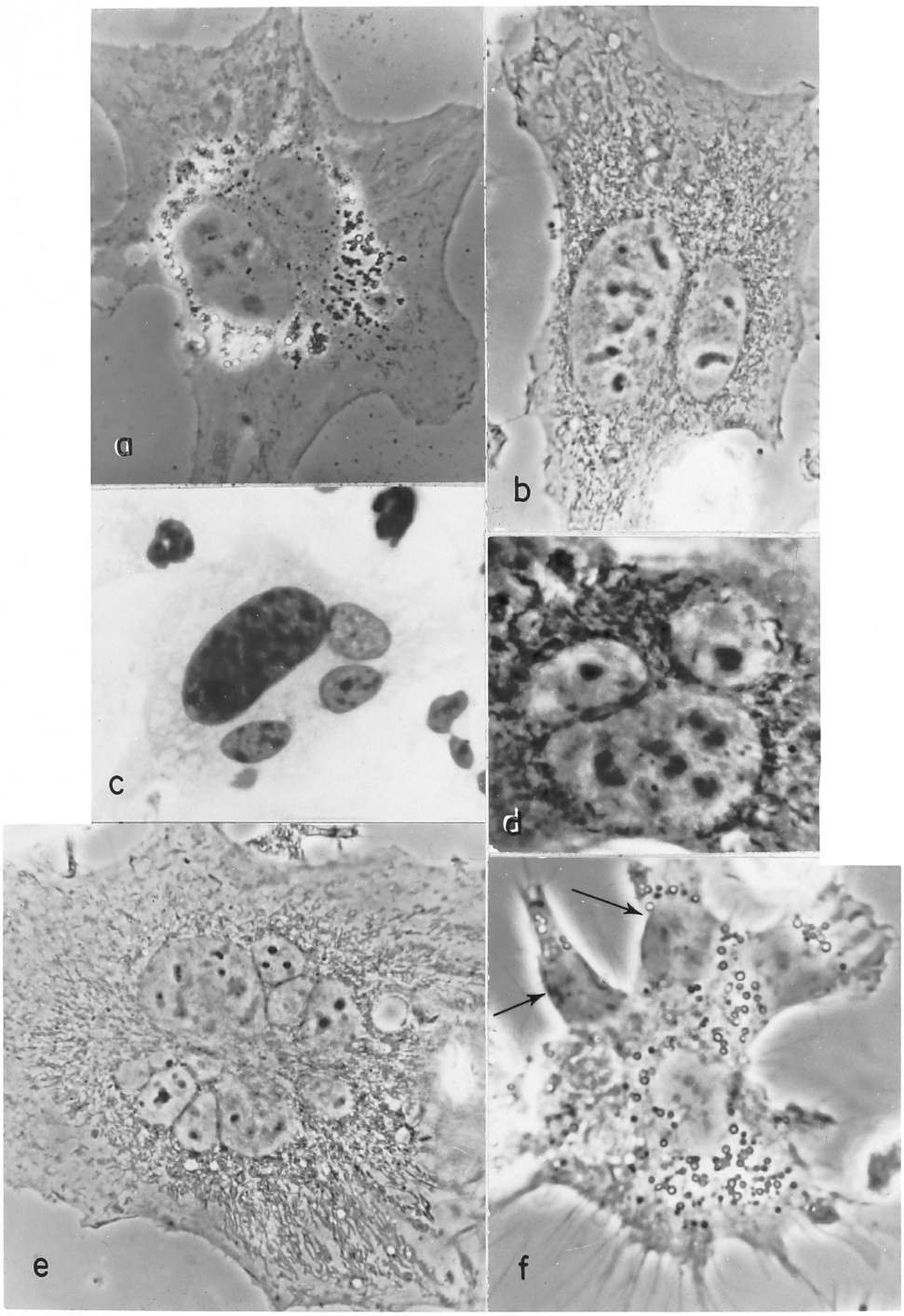
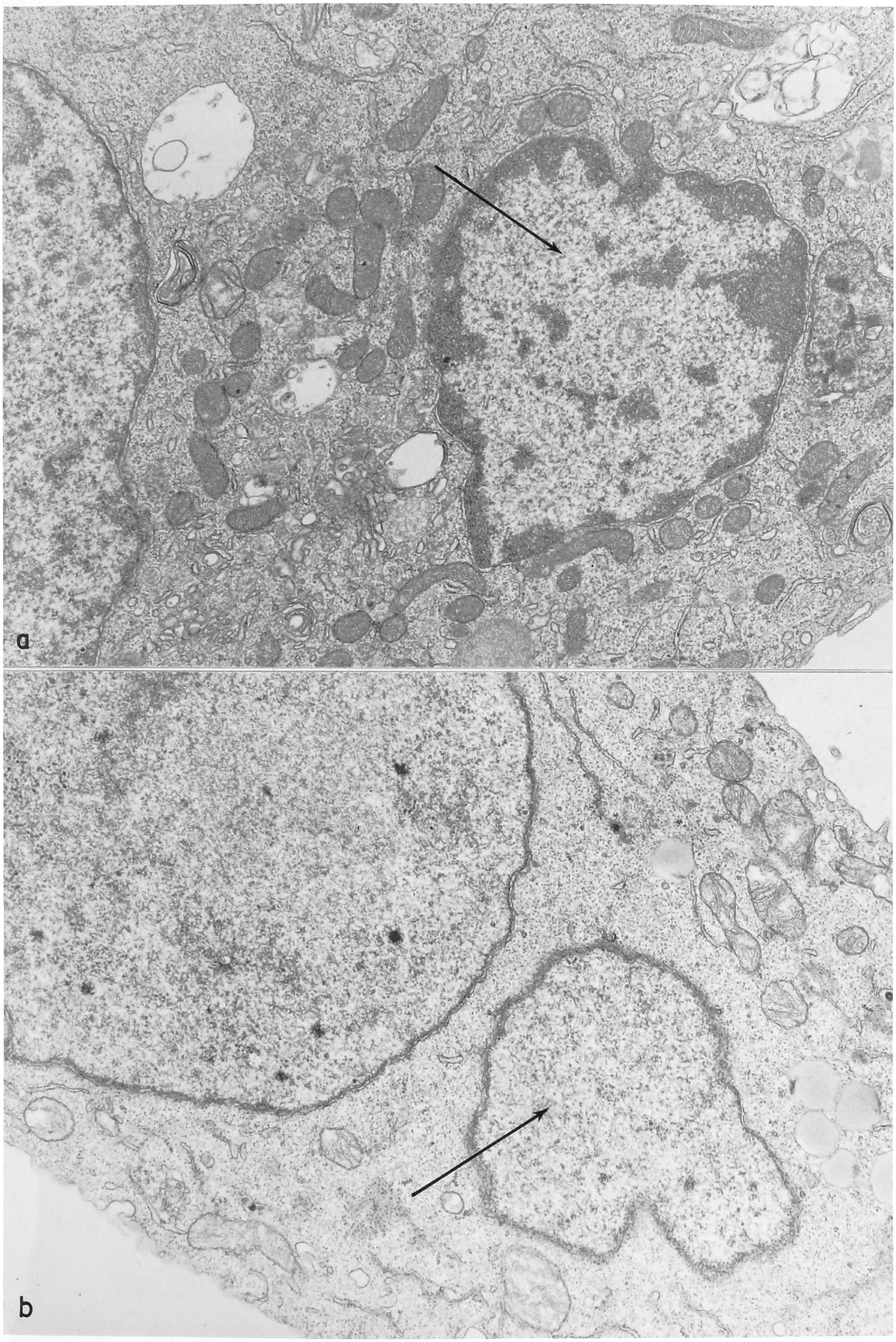

a
b
c
d
e
f

Figure 4

Electron micrographs showing the structure of the macrophage nucleus at different times after fusion with melanoma cells. (Courtesy Professor J. G. Hirsch)

Fig. 4a. A 1:1 heterokaryon, one hour after fusion. The macrophage nucleus (arrow) has a characteristic rim of heterochromatin, which is not present in the melanoma nucleus. x 15,000.

Fig. 4b. A 1:1 heterokaryon, 18 hours after fusion. The macrophage nucleus (arrow) has only dispersed chromatin. x 15,000.


a
b

The formation of macrophage homokaryons was also directly observed in living cells by time lapse cinematography and confirmed the sequence of events described. A bridge formed between adjacent cells within minutes of adding the virus. The refractile lipid droplets, characteristic of macrophages which have been cultivated in the presence of high serum concentration, served as a useful indicator of cytoplasm mixing. The speed with which cytoplasmic mixing and cell reorganisation occurred varied considerably. Fusion was often observed within 10 minutes and many homokaryons were fully reorganised by 60 minutes, although homokaryons with 4 or more macrophage nuclei could take as long as 3 hours to reach this stage. No further fusion occurred.

There was no morphologic evidence in either living or fixed cells, that Sendai virus had a deleterious effect on cell function. Even in the early "scrambled" phase the macrophages pinocytosed actively and displayed prominent ruffling of their membranes.

Unfused macrophages showed acid phosphatase reactive granules, especially after cultivation in serum rich medium. The macrophage homokaryons were strongly reactive at the time of fusion and became progressively more reactive during the course of further cultivation in vitro. Heterokaryons showed some reactivity immediately after fusion, but this disappeared over the next day or so.

After several days' cultivation the heterokaryons resembled melanoma cells in morphology. The refractile lipid droplets of macrophage origin disappeared from the heterokaryons, whereas macrophage homokaryons contained progressively more lipid droplets with prolonged cultivation.

The Fate of Fused Cells. In order to determine whether macrophage-melanoma cell heterokaryons could actually divide, 1:1

heterokaryons, prepared by trypsinization, were observed individually. Unfused melanoma cells still present in these preparations usually divided within 1 to 2 days and formed vigorously growing clones. In 3 separate experiments more than 100 1:1 heterokaryons were observed. Of these, 30 to 80%, depending on the particular experiment, yielded pairs of descendants. This usually occurred 2 to 4 days after fusion and resulted in cells morphologically indistinguishable from normal melanoma cells. In some instances heterokaryons were seen in mitosis. Since most heterokaryons eventually entered mitosis there were none left as such by the seventh day after fusion.

Fewer cells went into a second division and in only 1 case could a third division be traced directly. At a time when the unfused melanoma cells had formed colonies of over a hundred cells most heterokaryons had produced only 2 to 8 progeny. The ability of these heterokaryons to divide and form clones was therefore severely reduced when compared with unfused melanoma cells.

Shortly after the formation of heterokaryons, the dextran sulfate vacuoles of macrophage origin were randomly distributed in the common cytoplasm. On the first day after fusion only macrophages and heterokaryons contained the marker. During the next few days a new population of marked cells appeared. These were morphologically indistinguishable from melanoma cells, were often found in pairs and represented the progeny of heterokaryons. By the end of a week there were no heterokaryons left in such preparations and an occasional colony of up to 10 marked "melanocytic" cells could be found. Fewer dextran sulfate vacuoles were present after each division.

It was therefore concluded from both direct observation and the dextran sulfate marker experiments that heterokaryons remained

viable as such for up to a week, but that they disappeared from the preparation as mitosis started on the second day after fusion. Their progeny were morphologically indistinguishable from normal, unfused melanoma cells except for their sluggish proliferation.

Discussion

The cultivation of peritoneal cells for 24 hours, prior to the addition of virus, resulted in a uniform well spread population of macrophages on the glass surface. This allowed more efficient fusion to occur and yielded large numbers of heterokaryons which could be recovered and studied in detail. In addition it aided the morphological and cytochemical evaluation of the early phases of fusion. The efficacy of surface fusion has been reported in other systems as well (Davidson, 1969).

A heterogeneous population of fused and unfused cells is initially present and includes heterokaryons and macrophage homokaryons formed from different numbers of macrophages. This makes it possible to analyse macrophage dosage effects, and to compare heterokaryons with homokaryons, as well as with unfused cells. Since all these cells have been exposed to the same virus treatment and culture conditions, built-in controls are available. By the same token, such a heterogeneous population makes it difficult to apply biochemical procedures and one has to resort mainly to techniques which can be applied to individual cells, such as microscopy, autoradiography and cytochemistry.

When cells are fused with virus each partner contributes a nucleus, a variety of cytoplasmic organelles and a plasma membrane to the newly formed homokaryon or heterokaryon. After an early phase in which random mixing of nuclear and cytoplasmic components

occurs, a striking reorganization of cellular architecture takes place. One result is the formation of a common centrosphere about which organelles become oriented. This process proceeds irrespective of cell origin and is apparent in both macrophage homokaryons and macrophage-melanoma cell heterokaryons. The disruption of organization with colcemid suggests a role for microtubules in the directed migration of organelles and is in keeping with the studies of Holmes and Choppin (1968) on the formation of syncytia.

Macrophage homokaryons remain viable for many days after fusion. Studies to be reported later show that they phagocytose more actively than unfused cells and are richer in surface ATPase and acid phosphatase. The vigorous function of these macrophage giant cells is of interest since similar cells are formed, in vivo, in many states of pathology (Roizman, 1962).

In contrast, when a macrophage is fused with a melanoma cell, it undergoes drastic changes in its behavior. The macrophage nucleus swells and its nucleoli become prominent. Macrophage lysosomes and lipid droplets disappear from heterokaryons whereas they accumulate in macrophage homokaryons during in vitro cultivation. We have not identified the mechanism by which these organelles disappear, nor have we determined the fate of less distinctive organelles, like mitochondria.

CHAPTER III

THE ACTIVATION OF MACROPHAGE DNA SYNTHESIS

Introduction

Harris and his coworkers (1966) showed that DNA and RNA synthesis could be activated in the nuclei of dormant cells after fusion with cells which actively make these nucleic acids. Subsequent studies documented the importance of nuclear swelling during the activation process, especially in relation to the increase in RNA synthesis (Harris, 1967).

When mouse peritoneal macrophages were fused with mouse melanoma cells the macrophage nuclei also became enlarged. We now consider the question of nucleic acid synthesis in these heterokaryons.

Materials and Methods

1. Cell cultivation and the method of virus induced cell fusion have been described. For the present experiments we either used one day old cultures of exponentially growing melanoma cells or synchronized melanoma cells, as indicated.

2. Synchronized melanoma cells were obtained by shaking off mitotic cells while growing in monolayer. Cultures of exponentially growing melanoma cells in Falcon T-75 flasks were washed with a solution of phosphate buffered saline lacking divalent cations and these washes discarded. The flasks were then shaken vigorously and the dislodged cells collected. These cells were spun down, carefully resuspended in a small volume of culture medium consisting of 199

and 20% newborn calf serum and 5×10^3 - 1×10^4 cells added to 1 day old macrophage-monolayers. Two T-75 flasks usually yielded enough cells to prepare 20 - 40 coverslips. After 1 - 2 hours incubation, to permit attachment of the melanoma cells, the preparations were washed gently and incubated further. The cocultivated cells were fused, in the usual way, at different times after replating the melanoma cells. Many doublets, cells in telophase, were seen soon after replating the melanoma cells.

3. Macromolecular Synthesis in Heterokaryons. Coverslip preparations of heterokaryons were examined at various times after fusion by the following procedures.

a) RNA synthesis was studied by autoradiography using tritiated uridine as tracer. Duplicate preparations were incubated for 20 minutes or 60 minutes in Eagle's minimal essential medium + 10% newborn calf serum (MM), which contained 10 μc/ml tritiated uridine. Coverslips were washed twice in MEM, fixed in methanol and processed for autoradiography. Grains were counted to quantitate RNA synthesis.

b) DNA synthesis. Duplicate coverslips were incubated for serial 2 hour pulses in MM containing 10 μc/ml tritiated thymidine, washed, fixed and processed for autoradiography. Deoxyribonuclease digestion of methanol fixed material removed the incorporated label completely. Nuclei were easily scored as positive or negative for DNA synthesis.

c) Protein synthesis. Coverslips were incubated in tritiated L-leucine, 10 μc/ml, for 60 minutes and then processed for autoradiography. Grains were counted to quantitate protein synthesis. In all cases, at least 25 1:1 heterokaryons were scored per coverslip. Unfused macrophages and melanoma cells served as controls in each preparation.

Autoradiography. Coverslips were treated with 0.3 N TCA for 60 minutes at 4°C, washed thoroughly and dried. They were coated with Ilford L4 emulsion, exposed for 7 to 10 days and subsequently developed, fixed and stained by a modified Giemsa method (v. Furth and Cohn, 1968).

Reagents and Isotopes. The materials used in these experiments were purchased from the following sources: Uridine-5 ^{3}H, 25.9 Ci/mM, Thymidine methyl ^{3}H, 2 Ci/mM, and L-leucine-4, 5 ^{3}H 44 Ci/mM, New England Nuclear Corp.; L4 Emulsion, Ilford, Essex, England; Deoxyribonuclease I, Worthington Biochemical Corp.

4. Chick red cell-melanoma cell heterokaryons were prepared as follows. The red cells of 11 or 12 day old chick embryos were washed twice in 199 M, resuspended in 199 M and pipetted onto glass coverslips ($1 \times 10^{6}/cm^{2}$). After 1 hr at room temperature, 1×10^{4} exponentially growing melanoma cells, suspended in 199 M, were added to each coverslip. After another hour incubation at 37°C the cells were treated with 500 hemagglutinating units of inactivated Sendai virus for 30 minutes, washed twice and cultivated in 199 M. DNA synthesis of heterokaryons was measured by radioautography, using 4 consecutive 2 hour pulses with ^{3}H thymidine, 1 hr after treatment with virus, and another pulse 18 - 20 hours after fusion.

5. The inhibitors actinomycin D and bromotubercidin were obtained from Dr. E. Reich; cycloheximide and streptovitacin A from Dr. S. Silverstein of The Rockefeller University. Concentrated stock solutions were stored at -20°C and thawed and diluted immediately before use.

The effect of these drugs on DNA, RNA and protein synthesis in

unfused cells was studied by measuring the incorporation of tritiated precursors into trichloracetic acid (TCA)-insoluble products. L-leucine, uridine, and thymidine were all used at 5 μc/ml in MM. Replicate cultures were prepared for these incorporation studies by cultivating 1.2 x 10^5 melanoma cells or 3 x 10^6 macrophages in 35 mm tissue culture dishes (Falcon Plastics) for 1 day in MM. The cells were washed and treated with each drug in the presence of the appropriate precursor. After drug treatments of varying duration, the cells were washed 3 x with MEM and incubated further in the presence of radioactive tracer. In other experiments the cumulative incorporation of precursor was measured only after washing out the inhibitor. At various times the incorporation into TCA-insoluble products was measured as follows. The cells were washed 2 x with ice cold saline, scraped in saline and precipitated with an equal volume of 10% TCA. The precipitates were washed 2 x in 2.5% TCA and dissolved in N NaOH. Aliquots were counted in Bray's solution in a Mark II liquid scintillation counter with an efficiency of 34%. The results were expressed as cpm per μg protein. The protein content of samples was determined by the method of Lowry and coworkers (1951).

Inhibitors were also used in cell fusion experiments, either preceding or following treatment with virus. In "pretreatment" experiments one cell partner was treated with a particular drug, washed 3 x and fused with untreated cells one hour later. Controls for these experiments always included pretreatment of the other parent, as well as cells fused without drug treatment. In "post treatment" experiments, the inhibitor was added to cocultivated cells either at the same time as the virus, or afterwards. After treatment with the drug the fused cells were washed 3 x and cultivated further in 199 M.

Inhibitors were also used in experiments with synchronized

melanoma cells, in which case the inhibitor was added at different times after replating the mitotic melanoma cells, i. e. at different stages of the melanoma cell cycle. The inhibitor was either added at the time of fusion, or two hours afterwards, and treatment was then maintained continuously until the end of the experiment.

6. Characterization of the Perchloric acid (PCA)-soluble Pool After Incubation in ^{3}H Thymidine. Macrophages or melanoma cells were cultivated in 60 mm Falcon plastic tissue culture dishes for one day. The cells were washed twice with warm phosphate buffered saline and exposed to 4 μc/ml ^{3}H thymidine, in MM, for four hours. After two washes in ice-cold saline the cells were scraped in saline and mixed with 5% ice-cold perchloric acid. After two washes with 1:3 ethanol: ether, the PCA-soluble fraction was neutralized with 6N KOH, to pH 5. 0. The perchlorate was spun down and the supernatant fraction concentrated in vacuo. The constituents were separated by high voltage paper electrophoresis at pH 3. 5, eluted from the paper and their radioactivity measured in a scintillation counter.

Results

1. DNA Synthesis in Macrophage-melanoma Cell Heterokaryons. Newly formed heterokaryons were exposed to consecutive two hour pulses with ^{3}H thymidine in order to measure DNA synthesis at different times after fusion. The results of representative experiments are illustrated in Figs. 5 and 6. The melanoma cells were growing exponentially at the time of fusion and, as shown in Fig. 5A, about 60 - 70% of the melanoma nuclei were labelled in heterokaryons throughout the course of the experiment. The macrophage nuclei inside the heterokaryons were not labelled at first. After a lag period of about three hours, the macrophage nuclei started to make DNA and about 70%

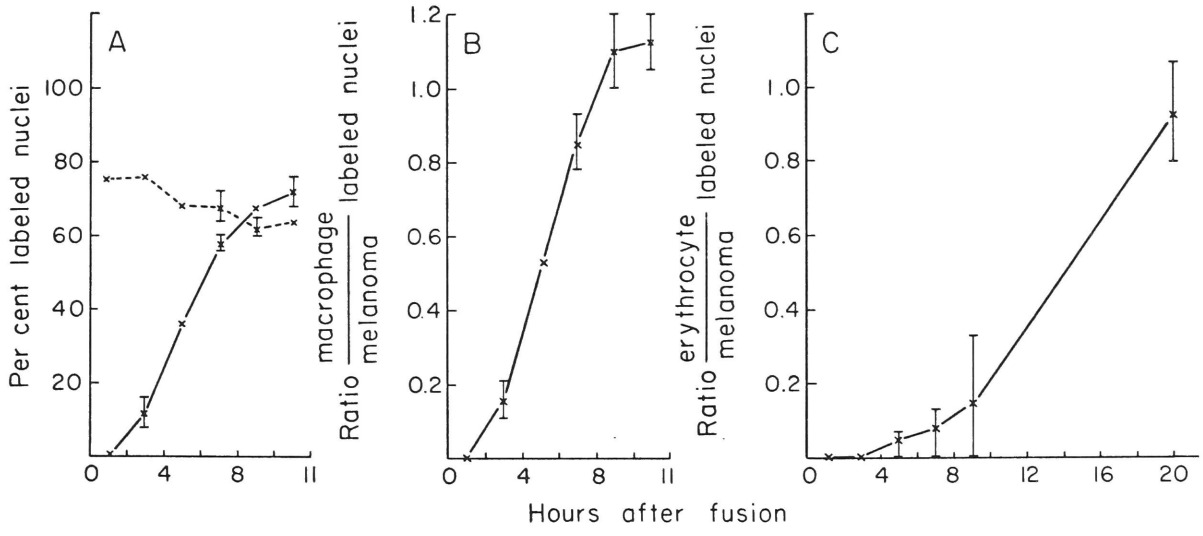


Figure 5. DNA synthesis in 1:1 heterokaryons at different times after fusion. A, B, macrophage-melanoma cell heterokaryons (x----x melanoma labelling, x——x macrophage labelling), C, erythrocyte-melanoma heterokaryons. The crosses represent the mean of three observations, the bars their range.

of them were labeled eight hours after fusion. The melanoma cell had therefore stimulated a wave of DNA synthesis in the dormant macrophage nuclei.

The kinetics of the initiation of macrophage DNA synthesis proved to be remarkably constant. An irreducible delay of 2 - 3 hours and the steep rise of the activation curve were characteristic features. Since 50 - 80% of macrophage nuclei invariably started to make DNA during the period 3 - 8 hours after fusion, it was possible to assay this parameter of macrophage nuclear activation reliably. The ratio of labelled macrophage to melanoma nuclei also provided a good indicator of the degree of activation, illustrated in Fig. 5B. The steep curve shows that the macrophages responded rapidly and the final ratio > 1 shows that all the macrophage nuclei could be induced to make DNA.

The activation of DNA synthesis was specific for heterokaryons. Unfused macrophages or macrophage homokaryons were never found labelled, thus excluding a nonspecific effect of cell fusion itself. Since the macrophage is capable of RNA and protein synthesis it was possible that the macrophage could actively inhibit melanoma DNA synthesis in heterokaryons. This is, however, not the case, as shown in Table III. The frequency of melanoma DNA synthesis was unaffected in heterokaryons containing as many as four macrophage nuclei. Moreover, the nuclei of multiple heterokaryons often labelled in synchrony, illustrated in Fig. 6(b).

2. DNA Synthesis in Chick Erythrocyte-melanoma Heterokaryons. The above studies suggested that macrophage nuclei were induced to make DNA sooner than were chick erythrocyte nuclei, placed in HeLa cytoplasm (Bolund et al., 1969). DNA synthesis in macrophage and erythrocyte nuclei was therefore compared in similar cytoplasm by fusing both types of cell with melanoma cells. The pooled results of

Fig. 6a. DNA synthesis by both nuclei of a 1:1 heterokaryon, Thymidine pulse 5 - 7 hours after fusion. Stained autoradiograph. x 1000.

Fig. 6b. Synchronous DNA synthesis by three macrophage and two melanoma nuclei in a heterokaryon. Thymidine pulse 5 - 7 hours after fusion. Note the absence of grains over the unfused macrophage nucleus. Stained autoradiograph. x 1000.

Fig. 6c. RNA synthesis in a 1:1 heterokaryon two hours after fusion. The macrophage nucleus (arrow) inside the heterokaryon has more grains and is larger than the adjacent unfused macrophage. Uridine pulse 20'. Stained autoradiograph. x 1000.

Fig. 6d. RNA synthesis in a 1:1 heterokaryon two hours after fusion. The macrophage was treated with 1 μg/ml actinomycin for one hour before fusion. The macrophage nucleus is enlarged and is heavily labelled. Uridine pulse 20'. Stained autoradiograph. x 1400.

Fig. 6e. RNA synthesis in a 1:1 heterokaryon two hours after fusion. The melanoma cell was treated with 5 μg/ml actinomycin for one hour before fusion. The melanoma nucleoli are small and round. Neither the melanoma nor the macrophage nucleus is labelled, whereas an unfused macrophage nearby is heavily labelled. Uridine pulse 60'. Stained autoradiograph. x 1400.

Fig. 6f. RNA synthesis in a 1:1 heterokaryon two hours after fusion. The macrophage was treated with 5 μg/ml actinomycin for one hour before fusion. The macrophage nucleus is virtually unlabelled. The melanoma nucleus shows reduced, but definite labelling and the nucleoli are smaller than usual, due to actinomycin cross-toxicity. Uridine pulse 60'. Stained autoradiograph. x 1200.

Fig. 6g. Melanoma cell after one hour treatment with 5 μg/ml bromotubercidin. The nucleoli appear as numerous, discrete, small bodies. Fixed in 1.25% glutaraldehyde. Phase contrast. x 1500.

Fig. 6h. Melanoma cell three hours after washout, following a four hour treatment with 5 μg/ml bromotubercidin. The nucleolar masses are larger than during treatment. Fixed in 1.25% glutaraldehyde. Phase contrast. x 1500.

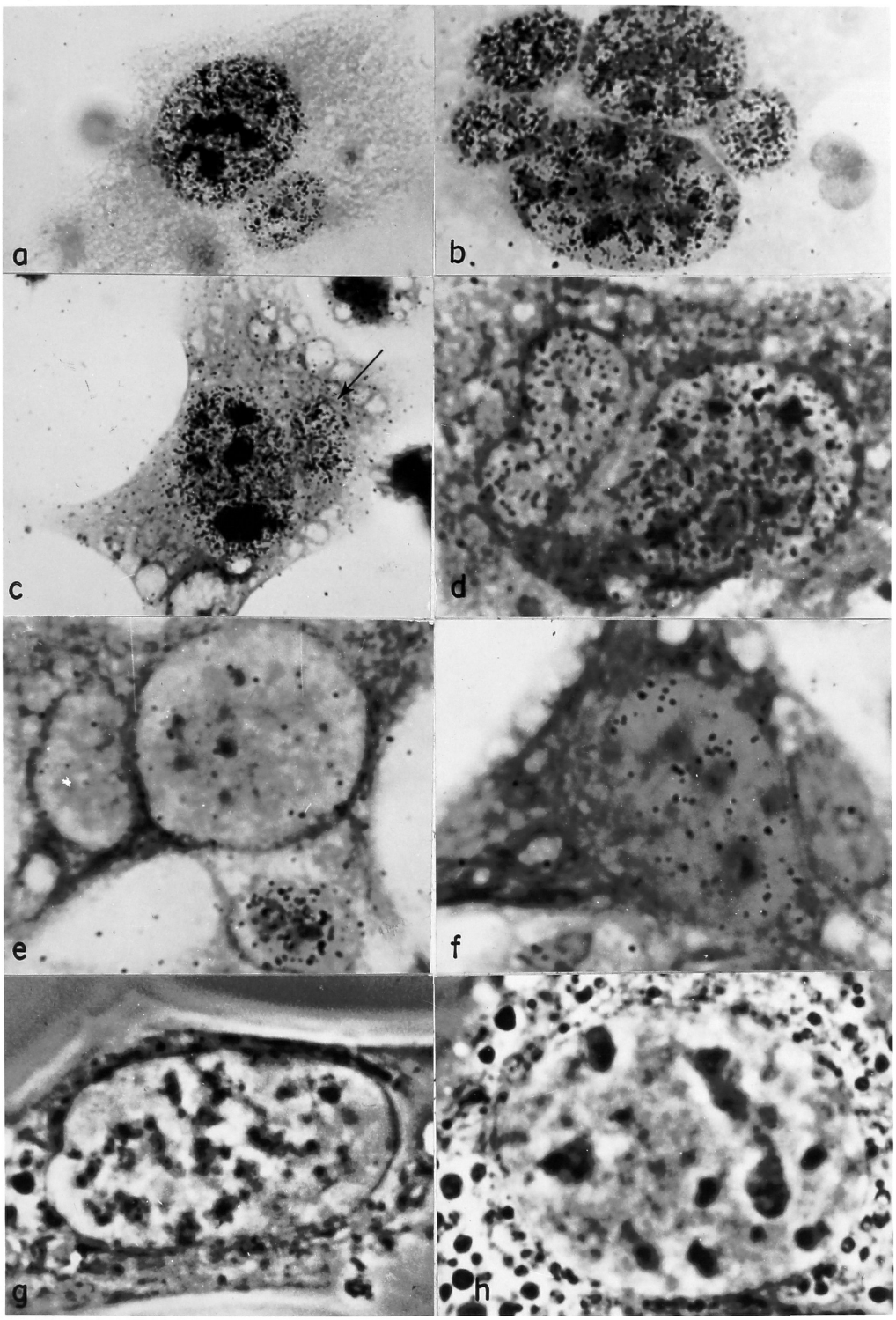

a
b.
c
d
e
f
g
h

TABLE III

The labelling pattern in multiple heterokaryons, following a ^{3}H thymidine pulse, 9-12 hours after fusion

Cell composition*	Total No. of cells scored	Labelling pattern†		Number of cells	Percent of all melanoma nuclei labelled
		Mac.	Mel.		
0:1	100		+	66	66
			-	34	
1:1	60				57
		+	+	30	
		-	+	4	
		+	-	7	
		-	-	19	
2:1	30				57
		++	+	14	
		+-	+	2	
		--	+	1	
		++	-	5	
		--	-	8	
3:1	30				60
		+++	+	16	
		++-	+	2	
		+--	-	1	
		++-	-	1	
		+++	-	3	
		---	-	7	
4:1	25				48
		++++	+	12	
		++++	-	1	
		----	-	12	

*The number of macrophage nuclei is listed first, i. e. a 3:1 cell has 3 macrophage nuclei and 1 melanoma nucleus.

†The macrophage nuclei are enumerated first, the melanoma nucleus last. E.G. ++-+, a 3:1 heterokaryon with 2 macrophage and 1 melanoma nucleus labelled.

three erythrocyte-melanoma cell fusion experiments are shown in Fig. 5C.

DNA synthesis in the chick nuclei started after a lag which varied from five hours after fusion, for a few nuclei, to more than nine hours after fusion, for most of the nuclei. This heterogeneity was present among different cells within the same experiment, as well as in different experiments. By 20 hours 48 - 70% of red cell nuclei were labelled. In parallel experiments, macrophage DNA synthesis had started by three hours and was found in 70% of 1:1 heterokaryons by seven hours. Sixty to 70% of melanoma nuclei were labelled in both types of heterokaryon throughout these experiments.

The macrophage nucleus therefore differs from the erythrocyte nucleus in the relative speed and homogeneity with which DNA synthesis is activated in heterokaryons and we conclude that the nature of the dormant nucleus is reflected in the kinetics of its activation.

3. RNA Synthesis in Heterokaryons. The unfused macrophages are able to make RNA, but heterokaryon formation results in an increase in macrophage labelling with uridine. This could reflect changes in precursor pool size, as well as a true stimulation of RNA synthesis. The kinetics of this process are shown in Table IV. Stimulated RNA synthesis can be detected within an hour of fusion and shows a two-fold increase by two hours (Fig. 6(c)). There is little further increase in macrophage RNA synthesis in heterokaryons relative to that in unfused macrophages. A lesser stimulation of RNA synthesis takes place in macrophage homokaryons.

After labelling with uridine for 20 minutes, grains were found over nucleoli as well as scattered over the rest of the nucleus. Fewer than 10% of the total grains were present over the cytoplasm of cells. After longer periods of labelling, or after a 40 minute "chase" with

TABLE IV

The incorporation of Uridine into macrophage nuclei

(No. of cells = 25, pulse = 20 min.)

No. of grains per macrophage nucleus

Hours after fusion	Unfused cells	1:1 Hetero-karyons	Binucleate Homokaryons (per nucleus)		Ratio $\frac{\text{heterokaryons}}{\text{unfused cells}}$
	Mean SD.	Mean SD.	Mean	SD.	
1	22.2±5.9	33.7±9.8	29.5	±9.0	1.5
2	18.2±4.8	33.8±8.6	25.8	±7.5	1.9
3	19.8±5.8	43.7±10.5	26.1	±10.4	2.2
4	19.2±5.7	43.4±7.7	24.6	±5.6	2.3
5	27.4±5.7	55.5±16.8	32.1	±9.8	2.0

non-radioactive uridine, there was an increase in cytoplasmic label of both fused and unfused cells. It was not possible to establish by means of grain counts, however, whether the macrophage nuclei contributed RNA to the heterokaryon cytoplasm.

Macrophage nuclei, therefore, make RNA at the time of fusion and the further stimulation in RNA synthesis in the heterokaryons precedes their DNA synthesis. Further experiments were undertaken to determine if macrophage DNA synthesis depended on heterokaryon RNA synthesis, and in particular, macrophage RNA synthesis. Two compounds, actinomycin D and bromotubercidin, were used to inhibit RNA synthesis.

4. The Effect of Actinomycin on RNA, DNA and Protein Synthesis. Actinomycin depressed RNA synthesis irreversibly in both melanoma cells and macrophages after treatment for one hour (Table V). 85 - 99% inhibition of RNA synthesis could be obtained, in both cells, in the dose range 1 - 5 μg/ml. When macrophages were exposed to actinomycin and ^{3}H uridine at the same time, the inhibition of uridine incorporation occurred somewhat more slowly than in melanoma cells, but, after a few hours macrophage RNA synthesis was affected more extensively. Many macrophages were dead five hours after treatment with 5 μg/ml.

Protein synthesis in both cell types was inhibited to a lesser extent than RNA synthesis, especially in the first hours of exposure and only one-third of protein synthesis was inhibited over the six hour period which followed treatment with 5 μg/ml. Melanoma DNA synthesis was unaffected for the first hour, but up to 2/3 inhibition followed treatment with 5 μg/ml.

5. Macrophage DNA Synthesis in Heterokaryons Treated with Actinomycin. The results of an experiment in which heterokaryons

TABLE V

The effect of actinomycin on RNA, protein and DNA synthesis in unfused cells*

TRACER	DOSE ACTINOMYCIN µg/ml.	TIME (hours)	INCORPORATION cpm/µg protein		PERCENT INHIBITION	
			MEL	MAC	MEL	MAC
^{3}H Uridine	0	1	150	150		
		3	508	462		
		6	948	908		
	0.1	1	74	90	51	40
		3	200	171	60	63
		6	422	235	51	74
	1.0	1	16	30	89	80
		3	63	50	88	89
		6	145	41	85	96
	5.0	1	7	21	95	85
		3	7	17	99	97
		6	25	17	97	98
^{3}H Leucine	0	1	32	11		
		3	97	31		
		6	188	52		
	0.1	1	N. D.	11	N. D.	0
		3	N. D.	32	N. D.	0
		6	N. D.	42	N. D.	19
	1.0	1	32	10	0	8
		3	84	24	13	22
		6	171	39	9	25
	5.0	1	34	10	0	8
		3	64	22	34	28
		6	118	33	37	36
^{3}H Thymidine	0	1	82			
		4	334			
		7	550			
	0.1	1	94		0	
		4	370		0	
		7	680		0	
	1.0	1	84		0	
		4	204		39	
		7	326		41	
	5.0	1	72		12	
		4	124		63	
		7	184		67	

*Cells were treated with actinomycin for 1 hr in the presence of radioactive precursor, washed 3x and incubated further in fresh precursor.

were treated with actinomycin, 1/2 - 1 1/2 hours after fusion, are illustrated in Fig. 7. Macrophage DNA synthesis, measured by radioautography, was prevented by 5 μg/ml and inhibited, to a lesser extent, by 0.5 μg/ml. A dose of 0.05 μg/ml had no effect. The number of labelled melanoma nuclei did not fall appreciably, even after 5 μg/ml, but the intensity of their labelling did diminish towards the end of the experiment.

Several morphologic changes were observed in preparations treated with actinomycin. The melanoma nucleoli were characteristically small and round (cf. Fig. 6(e)). There was considerable macrophage cell death after treatment with 5 μg/ml, but heterokaryons survived, like the unfused melanoma cells. The swelling of the macrophage nuclei in these heterokaryons was unaffected by actinomycin treatment.

The initiation of macrophage DNA synthesis was therefore sensitive to actinomycin treatment started one-half hour after fusion. The relationship between the timing of cell fusion and the period of actinomycin sensitivity was examined by treating cocultivated cells with actinomycin at different times, before or after fusion (Fig. 8).

Macrophage DNA synthesis was most severely depressed when the cells were treated with actinomycin two hours before fusion, but the effect was still striking when actinomycin and viral treatment coincided. Macrophage DNA synthesis was affected to a lesser degree when drug treatment was delayed until one - three hours after fusion, and no effect occurred at four hours. Fifty to 60% of melanoma nuclei were labelled in all groups. Cell fusion itself was unaffected by actinomycin treatment.

Low doses of actinomycin D have been reported to inhibit mainly ribosomal RNA synthesis (Perry, 1964). The selective effects of

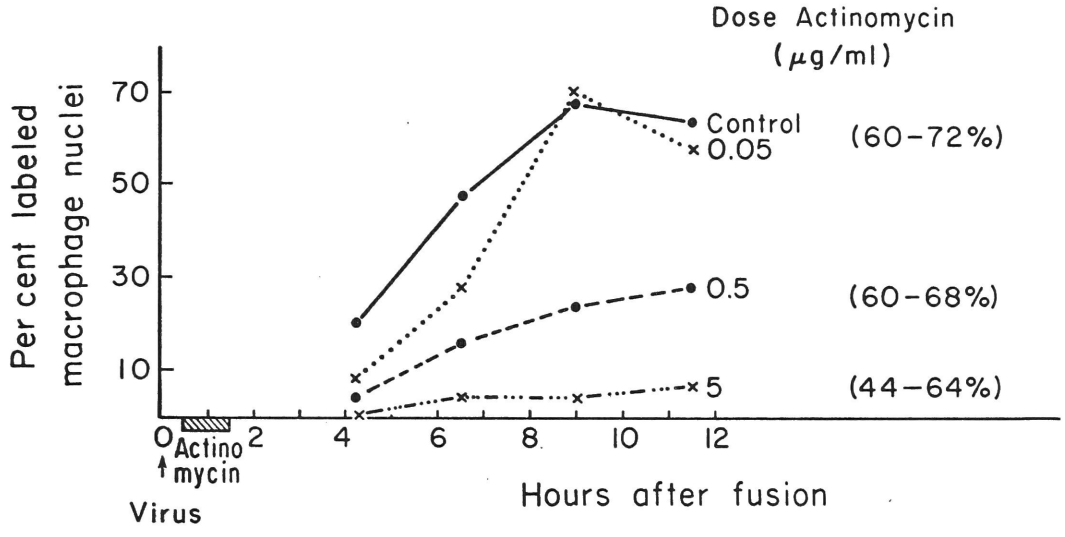


Figure 7. The effect of actinomycin treatment on the initiation of macrophage DNA synthesis in 1:1 heterokaryons. Percent labelled melanoma nuclei in brackets.

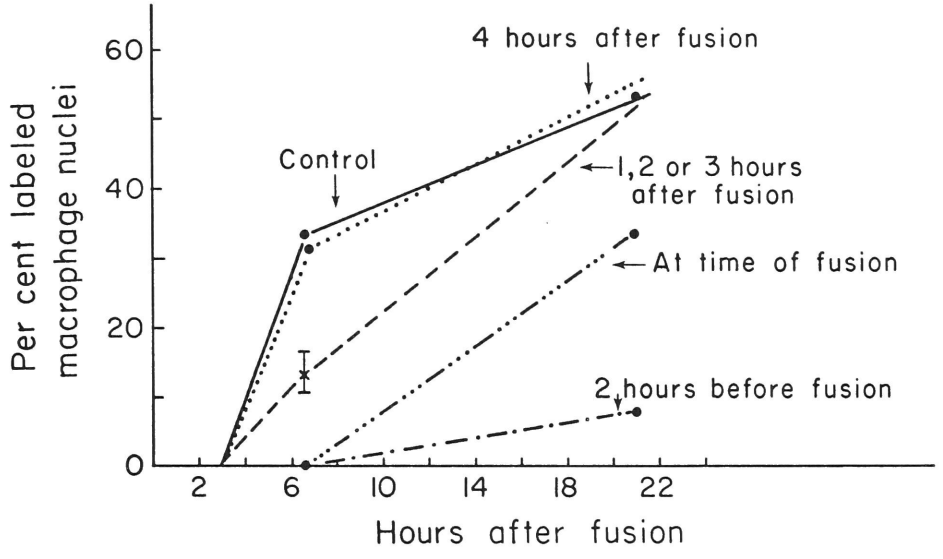


Figure 8. Macrophage DNA synthesis in 1:1 heterokaryons following actinomycin treatment at different times. Groups of coverslips were treated with 5 μg/ml actinomycin at hourly intervals, starting two hours before fusion and ending five hours after fusion. After an hour's treatment the preparations were washed 3 x and cultivated further. All groups exposed to ^{3}H thymidine 5 - 7 and 7 - 21 hours after fusion.

smaller doses of actinomycin on macrophage DNA synthesis and on melanoma RNA synthesis were therefore compared. This comparison rests on the assumption, to be substantiated later, that the melanoma cell provides all the RNA necessary for macrophage DNA synthesis to be achieved.

As can be seen in Fig. 9, macrophage DNA synthesis in heterokaryons was unaffected by less than 0.1 μg actinomycin per ml, a dose which inhibited 56% of melanoma cell RNA synthesis and which brought about the characteristic nucleolar changes. At a higher dose, both macrophage DNA and RNA synthesis were progressively inhibited. The number of labelled melanoma nuclei in heterokaryons varied between 63 and 83% and was undiminished by treatment.

This experiment showed that macrophage DNA synthesis could proceed independently of a large proportion of total RNA synthesis, presumably mainly ribosomal RNA, but depended on a species of RNA inhibited by > 0.1 μg/ml actinomycin.

6. Selective Inhibition of RNA Synthesis Before Fusion. To answer the question whether one or both nuclei of a heterokaryon contribute RNA necessary for macrophage DNA synthesis, each cell was treated with an inhibitor of RNA synthesis before fusion with its untreated partner. The diffusion of drug, in such experiments, from the treated nucleus of a heterokaryon to the untreated nucleus will be referred to as cross-toxicity. The extent to which this occurred in pretreatment experiments was evaluated from nucleolar morphology and by radioautography.

A. Actinomycin treatment before cell fusion. The actinomycin pretreatment experiments made it possible to dissociate DNA and RNA synthesis in the macrophage nucleus of heterokaryons. After

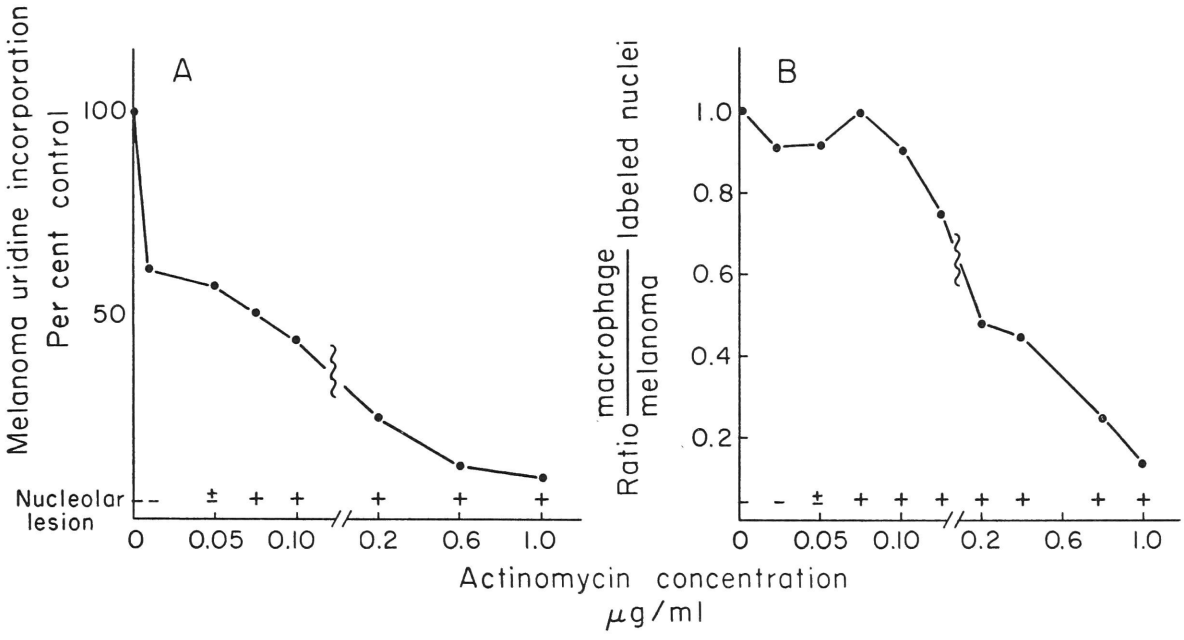


Figure 9. The effect of different doses of actinomycin on (A) RNA synthesis in unfused melanoma cells and (B) the activation of macrophage DNA synthesis in 1:1 heterokaryons. (A) Melanoma cells treated for one hour; incorporation ^{3}H uridine into TCA-insoluble product measured over four hours. (B) Cocultivated cells treated one hour before fusion. Radioautography, ^{3}H thymidine 1 - 7 hours after fusion. Melanoma nucleoli indicate morphologic effect of actinomycin.

macrophage pretreatment (5 μg/ml) RNA synthesis was reduced, before and after fusion, while DNA synthesis was unaffected (Fig. 10). Macrophage RNA synthesis amounted to less than 20% of that found in untreated heterokaryons during the first four hours after fusion. Cross-toxicity depressed melanoma RNA synthesis in these heterokaryons only moderately, 50 - 70% of control grain counts, illustrated in Fig. 6(f). These experiments suggested that macrophage RNA synthesis was not important for its own DNA synthesis.

When melanoma cells were pretreated (1 μg/ml) the macrophage nuclei made no DNA after fusion. Severe cross-toxicity abolished macrophage as well as melanoma RNA synthesis (Fig. 6(e)) so that melanoma pretreatment did not achieve a selective effect on the melanoma nucleus.

Cross-toxicity from the pretreated macrophage nucleus to the melanoma nucleus of a heterokaryon became more severe (20 - 30% of control grain counts) after pretreating macrophages with 10 μg/ml actinomycin and was then associated with some depression of macrophage DNA synthesis. When macrophages were pretreated with 1 μg/ml actinomycin the macrophages made no RNA before fusion, but were stimulated after fusion to make as much RNA as in untreated heterokaryons (Fig. 6(d)) showing that the macrophage nucleus became less susceptible to actinomycin after fusion.

B. Bromotubercidin treatment of cells. Bromotubercidin was used to distinguish between melanoma and heterokaryon RNA synthesis. This compound is an adenosine analogue which is incorporated into RNA and depresses RNA synthesis reversibly (Reich and Brdar, personal communication). It was therefore likely that melanoma cell pretreatment with bromotubercidin would not affect macrophage RNA synthesis in heterokaryons.

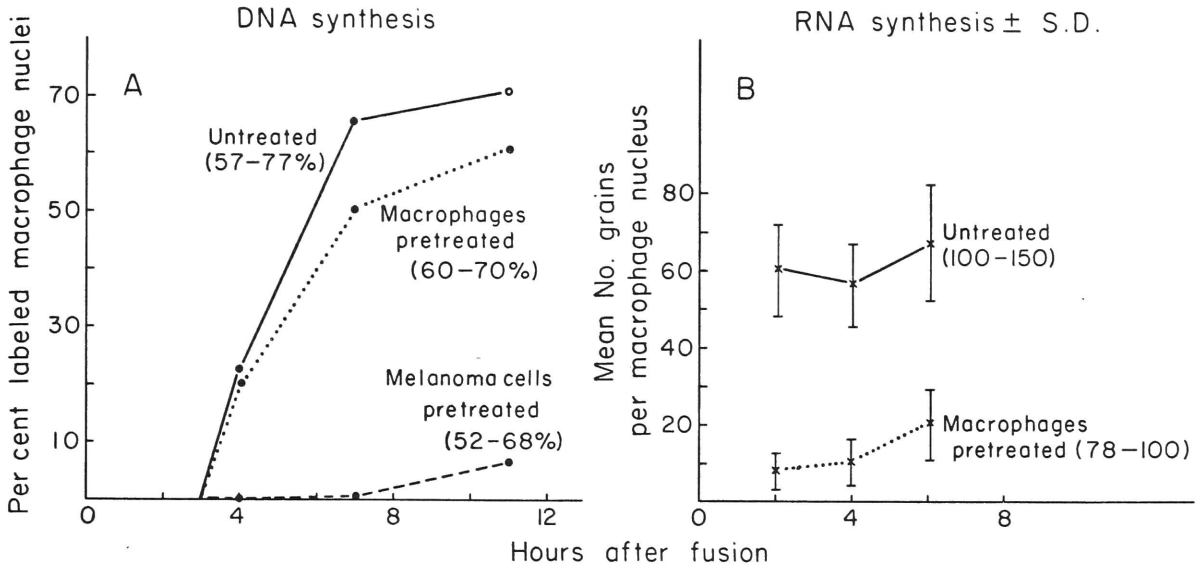


Figure 10. Macrophage DNA and RNA synthesis in 1:1 heterokaryons after treating either melanoma cells or macrophages with 5 μg/ml actinomycin for one hour before fusion. Melanoma label is given in brackets.

I. The effects of bromotubercidin on unfused cells. Bromotubercidin depressed 84% of melanoma cell RNA synthesis (Table VI). RNA synthesis recovered rapidly after one hour treatment and somewhat more gradually after five or nine hour treatment. Following a six hour period of treatment melanoma RNA synthesis recovered rapidly over the next eight hours, from 19 to 56% of control total RNA synthesis. Protein and DNA synthesis were relatively unaffected early, but declined later from 68 to 55%, and from 75 to 54% respectively. Bromotubercidin treatment caused a characteristic fragmentation of melanoma nucleoli which was fully reversible (Fig. 6(g, h)).

After four hour treatment macrophages became rounded up, making 12% of the control RNA. When bromotubercidin was removed the macrophages became well spread again and RNA synthesis proceeded at the same rate as in untreated cells. Protein synthesis was little affected by these conditions (68 - 75% of control).

These experiments showed that bromotubercidin could suppress RNA synthesis reversibly in melanoma cells and macrophages for several hours, without extensive depression of protein or DNA synthesis.

II. Bromotubercidin treatment before cell fusion. Figure 11 shows macrophage DNA synthesis in heterokaryons after seven hour pretreatment of melanoma cells or macrophages with bromotubercidin. After melanoma cell pretreatment the initiation of macrophage DNA synthesis was delayed until ten hours after fusion. 40 - 65% of the melanoma nuclei were labelled throughout this experiment, though often less heavily than in untreated controls.

RNA synthesis was evaluated by radioautography one - two hours after fusion. Treated melanoma cells had 30 - 50% as many grains

TABLE VI

The effect of Bromotubercidin treatment (5 μg/ml) on biosynthesis in unfused cells

A. The rate of RNA synthesis in melanoma cells treated with bromotubercidin for various times, based on 3 determinations at hourly intervals.

Treatment	Rate of incorporation of ^{3}H uridine cpm/μg protein/hour	Percent control
Nil	133	
During treatment (1, 5 or 9 hrs)	22	16
After treatment for:		
1 hour	180	132
5 "	110	83
9 "	100	75

B. Melanoma RNA, DNA and protein synthesis, after 6 hr treatment and further cultivation in the absence of bromotubercidin.

TRACER	HOURS AFTER STARTING TREATMENT	INCORPORATION cpm/μg prot. Control	Treated	PERCENT CONTROL
^{3}H Uridine	6	795	54	19
	8	1000	395	39
	10	1295	604	47
	12	1521	1000	56
^{3}H Thymidine	6	475	355	75
	8	646	490	75
	10	808	500	62
	12	1008	521	51
	14	1132	630	54
^{3}H Leucine	6	220	150	68
	8	295	174	59
	10	362	205	57
	12	440	238	55

C. Macrophage RNA and protein synthesis after 4 hr treatment and further cultivation in the absence of bromotubercidin.

TRACER	HOURS AFTER STARTING TREATMENT	INCORPORATION cpm/μg prot. Control	Treated	PERCENT CONTROL
^{3}H Uridine	4	731	86	12
	5	868	334	39
	6	1051	500	48
^{3}H Leucine	4	25	117	68
	5	32	21	66
	6	37	28	75

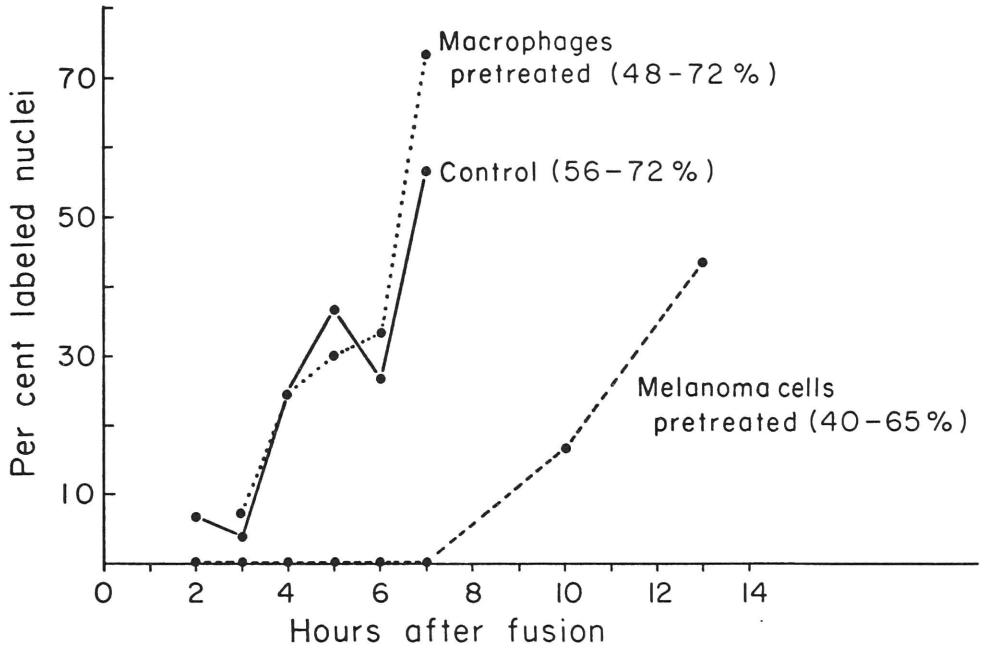


Figure 11. Macrophage DNA synthesis in 1:1 heterokaryons after treating either melanoma cells or macrophages with 5 μg/ml bromotubercidin for seven hours before fusion. Percent labelled melanoma nuclei given in brackets.

as untreated melanoma cells, indicating that melanoma cell RNA synthesis had not yet fully recovered. The macrophage nuclei of heterokaryons derived from pretreated melanoma cells had 81% as many grains as those in untreated controls (13.7 ± 4.9 cf 16.8 ± 9.0). Cross-toxicity had therefore not occurred in these cells. Shorter pretreatment of melanoma cells, for five or three hours, caused a similar delay in macrophage DNA synthesis, but the effect was reversed more rapidly. After one hour pretreatment no effect on macrophage DNA synthesis could be detected.

These experiments indicated that inhibition of melanoma cell RNA synthesis with bromotubercidin reversibly blocked macrophage DNA synthesis without affecting macrophage RNA synthesis. Melanoma RNA synthesis, by itself, was therefore essential for macrophage DNA synthesis and macrophage RNA synthesis could not substitute for the melanoma cell in this respect.

After macrophage pretreatment, heterokaryon DNA synthesis was indistinguishable from the control (Fig. 11). The treated macrophage nuclei in heterokaryons had 61% as many RNA grains as those in untreated controls (11.0 ± 5.6 cf 16.8 ± 9.0). Melanoma nuclear grains and morphology showed that no cross-toxicity had occurred from the treated macrophage nucleus.

These findings are also compatible with the previous conclusion that macrophage RNA synthesis is not required for DNA synthesis.

7. Protein Synthesis and Macrophage DNA Activation. Table VII shows the effect of cycloheximide on protein, RNA and DNA synthesis in unfused cells. 91 - 97% of protein synthesis was inhibited by three hours treatment with 1 - 5 μg/ml, but this effect was reversible for both melanoma cells and macrophages. Melanoma cell DNA synthesis was depressed 48 - 73% by cycloheximide treatment and RNA

TABLE VII

The effect of cycloheximide on protein, RNA and DNA synthesis in unfused cells*

Tracer	Dose Cycloheximide μg/ml.	Time Hours after start of Cycloheximide treatment	Incorporation cpm/μg protein MEL	MAC	Per cent inhibition MEL	MAC
^{3}H Leucine	0	3	160	84		
		4	215	128		
		6	320	180		
	0.1	3	40	38	75	55
		4	110	80	49	38
		6	205	160	36	11
	1.0	3	9	78	94	91
		4	66	34	63	73
		6	155	68	52	62
	5.0	3	6	2.8	96	97
		4	50	34	52	73
		6	135	68	60	62
^{3}H Uridine	0	3	230	310		
		5	430	420		
		7	608	600		
	0.1	3	160	387	30	-25
		5	355	400	17	5
		7	600	500	1	17
	1.0	3	137	226	40	27
		5	301	255	30	39
		7	580	390	5	35
	5.0	3	135	220	41	29
		5	250	230	42	45
		7	432	390	30	35
^{3}H Thymidine	0	3	170			
		5	250			
		7	361		—	
	0.1	3	95		44	
		5	142		43	
		7	206		43	
	1.0	3	79		54	
		5	66		73	
		7	125		65	

*Cells were treated with cycloheximide for 3 hrs. in the presence of radioactive precursor, washed 3x and then incubated in fresh precursor without drug.

synthesis inhibited to a maximum value of 45% .

Figure 12A illustrates the effect of cycloheximide on the activation of macrophage DNA synthesis in heterokaryons. Cycloheximide depressed macrophage DNA synthesis in a dose related fashion. 60 - 72% of melanoma cell nuclei in these heterokaryons incorporated thymidine, whether the cells had been treated with cycloheximide or not. Some macrophage nuclei which made no DNA as a result of cycloheximide treatment were nevertheless enlarged.

To find out which cell made the proteins for macrophage DNA synthesis each cell was treated, before fusion, with streptovitacin A, an irreversible inhibitor of protein synthesis. Table VIII shows the effect of streptovitacin A pretreatment on protein, RNA and DNA synthesis in unfused cells. Treatment with 2 μg/ml streptovitacin could suppress 80% of protein synthesis without reversal over four hours. Maximal inhibition of protein synthesis was associated with 74% inhibition of DNA and 45% inhibition of RNA synthesis.

The effect of streptovitacin pretreatment before cell fusion is shown in Fig. 12B. Macrophage DNA synthesis was unaffected by pretreating macrophages with 2 μg/ml streptovitacin, but melanoma cell pretreatment, 0.5 - 2.0 μg/ml, caused a dose-related depression of macrophage DNA synthesis. 52 - 72% of melanoma cell nuclei were labelled with thymidine at all times.

After streptovitacin pretreatment, fused preparations were incubated with ^{3}H leucine to measure protein synthesis (Table IX). In the untreated control macrophage nuclei, inside heterokaryons were more heavily labelled than those in unfused cells. After streptovitacin treatment of macrophages heterokaryon grain counts were undiminished, but after melanoma cell pretreatment, the nuclei and cytoplasm of

TABLE VIII

The effect of streptovitacin A on protein, RNA and DNA synthesis in unfused cells*

Tracer	Dose streptovitacin µg/ml.	Hours after washing out drug	Incorporation cpm/µg protein MEL	MAC	Per cent inhibition MEL	MAC
^{3}H Leucine	0	2	108	180		
		4	222	300		
	0.1	2	76	84	30	53
		4	156	169	30	43
	0.5	2	44	N.D.	59	N.D.
		4	80	N.D.	64	N.D.
	1.0	2	26	26	76	86
		4	66	64	70	79
	2.0	2	18	N.D.	84	N.D.
		4	46	N.D.	79	N.D.
	5.0	2	16	17	85	90
		4	42	40	81	87
	10.0	2	12	N.D.	89	N.D.
		4	38	N.D.	83	N.D.
^{3}H Uridine	0	2	220	194		
		4	444	400		
	0.1	2	212	294	4	-50
		4	380	478	14	-20
	0.5	2	182	N.D.	17	N.D.
		4	300	N.D.	32	N.D.
	2.0, 5.0, 10.0	2	124	202	44	-1
		4	244	248	45	38
^{3}H Thymidine	0	4	170			
		7	320			
	0.1	4	175		8	
		7	290		9	
	1.0	4	70		63	
		7	110		66	
	2.0, 10.0	4	50		74	
		7	100		69	

*Cells were treated with streptovitacin for 1 hour, washed 3 x and incubated in radioactive precursor without inhibitor.

Protein synthesis and macrophage DNA synthesis

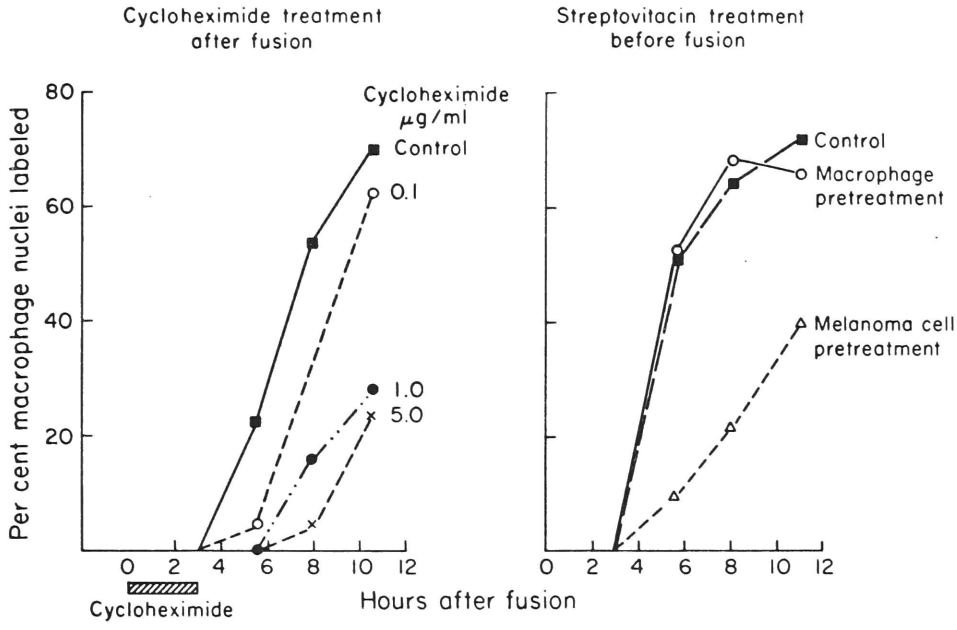


Figure 12

TABLE IX

Leucine labelling after Streptovitacin pretreatment

Pretreatment	1:1 Heterokaryons					Unfused macrophages			
	Melanoma nucleus		Macrophage nucleus		Cytoplasm	Nucleus		Cytoplasm	
	Mean	S. E.	Mean	S. E.		Mean	S. E.	Mean	S. E.
Control	84. 4	9. 9	31. 5	3. 3	>200	4. 5	. 76	15. 3	2. 0
Macrophages	96. 9	3. 3	34. 6	2. 2	>200	1. 3	. 56	5. 0	1. 0
Melanoma cells	33. 0	3. 8	12. 3	1. 9	75. 1	5. 5	. 87	19. 6	2. 2

(Melanoma cells or macrophages were treated with 2 μg/ml streptovitacin A, for one hour before fusion. Preparations were exposed to ^{3}H L Leucine, 10 μc/ml, 1 - 2 1/2 hours after fusion. Grain counts for 20 cells.

heterokaryons showed reduced labeling.

Macrophage DNA synthesis could therefore be depressed by selective inhibition of melanoma cell protein synthesis.

8. Fusion Studies with Synchronized Melanoma Cells. When melanoma cells in mitosis were replated on macrophage monolayers they entered S in a synchronous fashion 11 hours later and 98% of melanoma cells labelled with thymidine four - six hours thereafter. The exit from S into G2 was less synchronous and the peak of mitosis occurred 23 hours after replating. The cell cycle after replating was thus considerably longer than the 12 hour doubling time observed during exponential growth of melanoma cells.

Figure 13 shows DNA synthesis in heterokaryons after fusing macrophages with synchronized melanoma cells at different times during the melanoma cell cycle. The onset of melanoma cell DNA synthesis was not affected by exposure to Sendai virus or by fusion with a macrophage. When the melanoma cells were in G1 at the time of fusion (Fig. 13A and B) macrophage DNA synthesis was delayed until after the onset of melanoma cell S. When fusion occurred late in melanoma G1 (13C) or in mid S (13D) macrophage DNA synthesis showed the basic three hour lag in initiation and then proceeded rapidly to involve 80 - 85% of the macrophage nuclei. As the melanoma cells reached the end of S (13E) fewer macrophage nuclei entered S after fusion, until the start of the next melanoma cell cycle (Fig. 13F).

These experiments showed clearly that macrophage DNA synthesis was under the control of the melanoma cell cycle. The macrophage nucleus, however, still lagged in its response even after fusion with melanoma cells in mid S.

Protein and RNA synthesis were next inhibited at different times

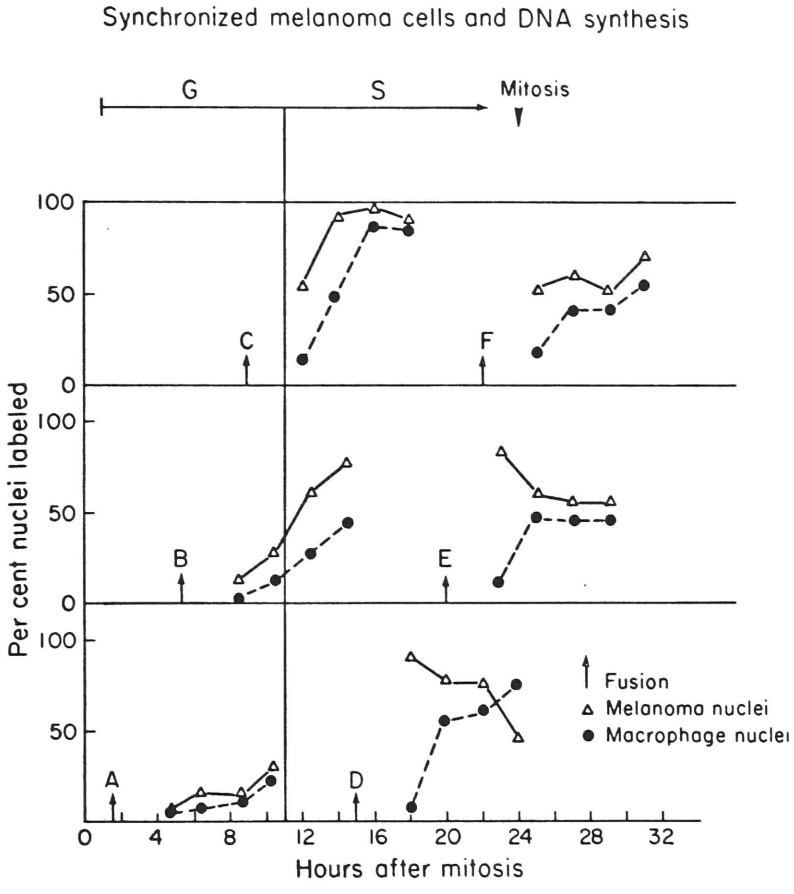

Synchronized melanoma cells and DNA synthesis
G
S
Mitosis
100
50
0
C
F
B
E
A
D
Per cent nuclei labeled
Fusion
Melanoma nuclei
Macrophage nuclei
0
4
8
12
16
20
24
28
32
Hours after mitosis

Figure 13

during the melanoma cell cycle (Table X). DNA synthesis was prevented in both the melanoma and macrophage nuclei if actinomycin or cycloheximide treatment was started in mid G_1 (four - seven hours). When inhibitor treatment was delayed till late G_1 (nine hours) more melanoma than macrophage nuclei initiated DNA synthesis. Both nuclei achieved maximum initiation of DNA synthesis if macrophages were fused with melanoma cells in S and inhibition delayed for two hours. However, when the heterokaryons were immediately treated with inhibitors of RNA or protein synthesis (fusion at 15 hours), the number of labelled macrophage nuclei was reduced selectively.

9. The PCA-soluble Products of ^{3}H Thymidine in Unfused Cells. It was by now apparent that macrophage DNA synthesis depended, in the main, on products made by the melanoma cell. Could unfused macrophages by themselves convert ^{3}H thymidine to thymidine triphosphate for incorporation into DNA? Macrophages and melanoma cells showed quite different profiles of their acid-soluble thymidine products (Fig. 14). Thymidine taken up by the macrophage remained unphosphorylated, whereas it was recovered mainly as thymidine triphosphate from the melanoma cells.

Discussion

DNA synthesis was stimulated in dormant macrophage nuclei by fusion with rapidly proliferating melanoma cells. This stimulation occurred even when several macrophages were fused with a single melanoma cell and often resulted in synchronous DNA synthesis in all the nuclei. The non-dividing mouse peritoneal macrophage, therefore, seems to lack elements which induce DNA synthesis rather than actively inhibiting this process (Harris, 1966).

Macrophage heterokaryons presented several advantages as a

TABLE X

DNA synthesis in macrophage-melanoma cell heterokaryons after inhibiting RNA and protein synthesis at different times during the melanoma cell cycle

Inhibitor	Time of fusion*	Start of inhibitor treatment**	Maximum % thymidine labelling in heterokaryon***	
	hours	hours	Macrophage nuclei	Melanoma nuclei
	9 (or earlier)	-	86	96
	15	-	60	70
Actinomycin (2 μg/ml)	2	4	0	6
	5	7	8	20
	7	9	40	70
	11	13	70	70
	15	15	20	73
Cycloheximide (5 μg/ml)	4	6	2	8
	7	9	44	84
	10	12	80	90
	15	15	37	87
Streptovitacin A (2 μg/ml)	15	15	33	73

*Mitotic melanoma cells replated on macrophage monolayers at time zero.

**Inhibitors were present continuously until the end of the experiment.

***2 hr. thymidine pulses until 21 hrs. after replating melanoma cells.

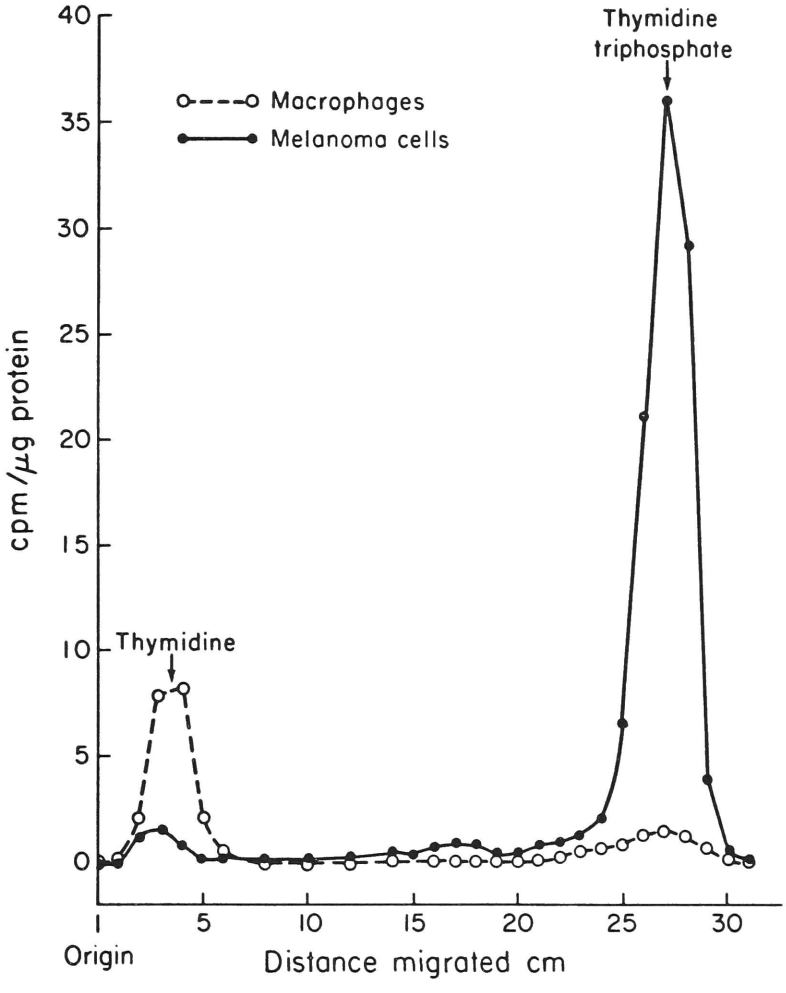


Figure 14. The PCA-soluble products of ^{3}H thymidine in unfused cells.

model system for DNA synthesis initiation. The unfused macrophages uniformly failed to make DNA until shortly after fusion with melanoma cells and the onset of DNA synthesis was synchronous, rapid and reproducible. It therefore became possible to study some of the requirements for DNA synthesis in the macrophage nucleus.

Macrophage DNA synthesis always lagged two - three hours behind that of the melanoma nucleus in the same cytoplasm and the chick erythrocyte nucleus responded even more slowly. The delay in onset of macrophage DNA synthesis is not an artifact of fusion, which is often complete in one-half hour. Moreover, HeLa nuclei in G_1 have been reported to start DNA synthesis within an hour of fusion with other HeLa cells which are already in S (Rao and Johnson, 1970a). The heterochromatin content of macrophage and erythrocyte nuclei could account for the different kinetics of initiating DNA synthesis in heterokaryons. DNA replication in heterochromatin can occur later than in euchromatin, even when present in the same nucleus (Prescott, 1970). The macrophage chromatin becomes more dispersed as the nucleus swells in a heterokaryon and probably undergoes changes in structure similar to those described by Bolund and his coworkers (1969), which make it competent to respond to cytoplasmic stimuli for DNA synthesis.

Actinomycin prevented initiation of macrophage DNA synthesis, most effectively when treatment of heterokaryons was started one-four hours prior to DNA synthesis. Although the nature and function of the RNA species which are involved in the initiation of DNA synthesis are completely obscure, higher doses of actinomycin were needed to prevent the initiation of DNA synthesis than to inhibit the bulk of RNA synthesis. These results are compatible with a model in which new RNA species, perhaps messenger RNAs, made some

hours prior to DNA synthesis, determine a new round of DNA replication. In this respect heterokaryons conform to other model systems for stimulated DNA synthesis, in which early changes in RNA synthesis take place (Baserga, 1968; Prescott, 1970). There is, however, a striking difference between other models and macrophage-melanoma heterokaryons in that the pre-replicative period is reduced from the usual 12 - 15 hours, or longer to 2 - 3 hours. This difference is presumably due to the direct use of melanoma products by the macrophage nucleus, bypassing many steps essential for DNA synthesis in unfused cells.

Evidence has been presented that the melanoma nucleus provides the RNA species necessary for macrophage DNA synthesis in heterokaryons. Bromotubercidin pretreatment of melanoma cells before fusion selectively inhibited macrophage DNA synthesis after fusion, without affecting its RNA synthesis. Other evidence also argues that the stimulation in macrophage RNA synthesis is not critical for subsequent DNA synthesis. Macrophage DNA synthesis is most sensitive to actinomycin treatment before macrophage RNA synthesis is much increased and macrophages which have been treated with high doses of actinomycin before fusion make DNA, but little RNA, afterwards.

The use of inhibitors to achieve selective effects in heterokaryons presents some interesting problems. Since the cell fusion process itself is independent of DNA, RNA or protein synthesis, it is possible to treat cells with various inhibitors before, as well as at the time of fusion. An important complication arises if the inhibitor diffuses from treated to untreated regions of a heterokaryon. Actinomycin, which is noncovalently bound to DNA, readily attacks untreated nuclei in heterokaryons (Reich and Goldberg, 1964). However, bromotubercidin is incorporated into RNA and does not give rise to such cross-toxicity.

Once the macrophage nucleus becomes activated in the heterokaryon it becomes less sensitive to the action of actinomycin. Macrophages which make no RNA after treatment with 1 μg/ml actinomycin showed unimpaired stimulation of RNA synthesis after fusion with untreated melanoma cells. Ringertz and his coworkers (1969) contend that RNA synthesis and the binding of actinomycin D vary in different cells in parallel with the functional state of their chromatin. Activation of a pretreated macrophage nucleus in a heterokaryon may make new sites, unoccupied by actinomycin, available for RNA synthesis.

The experiments with cycloheximide showed that protein synthesis is required for the macrophage nucleus to enter S; the studies with streptovitacin A suggested that these proteins came from the melanoma cell. These two compounds are closely related in structure and mode of action, although the detailed mechanism of inhibition has not been determined (Felicetti et al., 1969; Ennis, 1968). In addition, streptovitacin A becomes irreversibly bound within cells.

Although macrophage and melanoma cell cytoplasm mix intimately after fusion, macrophage pretreatment with streptovitacin did not inhibit heterokaryon protein or DNA synthesis. Its selective action argues against free diffusion of the drug within the fused cell. Another irreversible inhibitor of protein synthesis emetine hydrochloride (Grollman, 1968) did abolish heterokaryon protein synthesis after macrophage pretreatment (unreported observations).

Studies with synchronized melanoma cells showed, in agreement with other heterokaryon findings (Rao and Johnson, 1970a) that the S phase overrides G_o. The novel aspects of the present study include the characteristic lag in macrophage response and the continued dependence on RNA and protein synthesis even at melanoma mid S.

Macrophage and melanoma cells differ in thymidine kinase

activity and thymidine triphosphate content after incubation in ^{3}H thymidine. This distinction is typical of resting and proliferating cells, but is not considered of primary importance in regulating DNA synthesis (Nordenskjöld et al., 1970).

All the evidence therefore suggests that the melanoma cell, in following its own cell cycle, provides RNA, proteins and precursors which act upon a relatively passive macrophage nucleus to induce DNA synthesis. It is likely that proteins, ions and DNA precursor enzymes are transported into the macrophage nucleus during the early phase of swelling. The lag in response of the macrophage nucleus suggests, however, that the DNA template must be prepared in some way for replication. The nature and duration of these changes may be related to the heterochromatin content of the macrophage nucleus. The macrophage-heterokaryon system does make it possible to separate the role of template and inducing signals in bringing about DNA synthesis in G_o cells.

CHAPTER IV

THE SURFACE PROPERTIES OF MACROPHAGE-MELANOMA CELL HETEROKARYONS

Introduction

Major differences exist in the specialized functions which the macrophage and melanoma cells are able to perform. Since the mechanisms by which cell specialization is achieved are not understood, the fate of such cell-specific functions in heterokaryons is of considerable interest.

Materials and Methods

1. Attachment and Ingestion of Sensitised Erythrocytes. Sheep red cells in Alsever's solution were obtained weekly from the Animal Blood Center, Syracuse, N. Y. Older red cells were not used, to avoid non-specific attachment to macrophages. The red cells were washed 3 x in normal saline and a 5% stock suspension prepared in 199. The concentration of red cells was determined by dissolving an aliquot in 0.1 N Na_2CO_3 and measuring its absorbancy at 541 mμ (Kabat and Mayer, 1961).

The cells were sensitised by incubating a 1% suspension of red cells with a 1/2,000 dilution of a hyperimmune rabbit anti-sheep red cell antiserum for 30 minutes at 37°C. This antiserum was a gift from Dr. M. Rabinovitch, of The Rockefeller University and contained mainly 7S antibody, at a titre of 1/5,760. The sensitised red cells were washed 2 x in 10 ml 199 and then resuspended as a 1 % suspension in 199. This reagent was stable for two days at 4°C.

Heterokaryon preparations were washed twice with 199 and exposed

to a 0.1% suspension of sensitised sheep red cells at room temperature for 15 minutes. During this time sensitised red cells attached selectively to the surface of macrophages. The preparations were then washed 4 x and incubated in 199 M for a further 30 minutes at 37°C, to permit complete ingestion of red cells. The cells were then washed 2 x in 199, fixed with glutaraldehyde and stained with toluidine blue, 0.1% in 30% methanol. Ingested and attached red cells, which stain distinctively by this procedure, were then counted. Unless stated otherwise, 100 cells were scored at random and the mean number of red cells ingested per macrophage used as an index of ingestion.

2. ATPase. The histochemical procedure was based on that of Farquhar and Palade (1966). Heterokaryon preparations were fixed with ice cold 1.25% glutaraldehyde in PD for 10 minutes. Coverslips were washed twice in water and then incubated at 37°C for 30 minutes in a substrate mixture containing 5 mM ATP, 5 mM $MgSO_4$, 100 mM NaCl, 30 mM KCl, 2.4 mM $Pb(NO_3)_2$ and 80 mM Tris maleate, pH 7.2. Coverslips were rinsed in water, washed with 1% acetic acid for 1 minute and treated with a dilute ammonium sulfide solution for 20 seconds. Preparations were mounted in water and examined by both phase contrast and bright field microscopy.

Control studies showed that no lead precipitate formed in the absence of Mg^{++} or ATP, or after the cells had been fixed in methanol.

Biochemical assay. Since there are pitfalls in the use of lead ions to demonstrate phosphatases (Moses and Rosenthal, 1968), care was taken to correlate histochemical investigation with biochemical studies performed in the absence of lead. Living cells were assayed for surface ATPase activity by measuring the release of inorganic phosphate ion (P_i) from ATP. Replicate preparations of macrophages (1×10^6 cells) were cultivated on 22 mm^2 coverglasses for one day. The cells

were washed twice and incubated at 37°C, for 20 minutes, in 1 ml of the same substrate mixture used in the histochemical procedure, except that $Pb(NO_3)_2$ was omitted. The incubation medium was added to 0.3 ml of ice cold 20% TCA containing activated charcoal. The charcoal adsorbed residual ATP quantitatively from the solutions and was then itself removed by centrifugation. The P_i content was assayed by the method of Chen, Toribara and Warner (1956). The cell monolayer was scraped from the coverslip in normal saline and its protein content measured by the method of Lowry and coworkers (1951). Results were expressed as P_i released per μg protein per minute.

The release of P_i was linear under the conditions of assay and the cells remained viable throughout the incubation period as judged by phase contrast microscopy and dye exclusion. Trichloracetic acid extraction of the cells after incubation showed that the cell bound P_i was insignificant compared with P_i released into the incubation medium.

The substrate specificity and ionic requirements were determined by using appropriate incubation mixtures. Tris-ATP was substituted for the disodium salt when necessary. Ouabain was used at a concentration of 0.1 mM.

Further observations were made to correlate the histochemical and biochemical analyses. It was found that glutaraldehyde fixation itself reduced detectable enzyme activity by 83%. When several different cell lines were examined for ATPase activity by both types of procedure it was found that the histochemical and biochemical procedures showed excellent agreement over a wide range of enzyme activity.

Reagents were obtained from the following sources. ATP, Sodium Salt, Mann Research Lab.; Tris Salt, Sigma Chemical Co.;

Sodium β-glycerophosphate, Eastman Kodak; Ouabain, Sigma Chemical Co.; Sodium β-naphthyl acid phosphate, Dajac Lab.; Cyclic AMP, Calbiochem; AMP2'3', Mann Research Lab.; ITP, ADP, GTP, CTP, AMP5' and glucose-6-phosphate were all obtained from the Nutritional Biochemical Corporation.

3. Enzymatic and Chemical Treatment of Heterokaryons. Exponentially growing melanoma cells were fused with macrophage monolayers in the usual way. The preparations were cultivated in 199 + 20% NBCS for one to two days after fusion, until most of the Fc receptor activity had been lost. The cells were washed 2 - 4 x in 199 and treated for 30 minutes at 37°C with the appropriate reagent. The treatment was stopped by gently removing the reagent and the cells were then handled in two ways, depending on the morphologic effect of the treatment. If melanoma cells and heterokaryons showed no detachment, the cells were washed 2 - 4 x in 199 and then immediately assayed for phagocytic activity, using a 1/3,000 dilution of antibody to coat the sheep red cells. If detachment did occur, the cells were either incubated further in 199 + 10% NBCS, in situ, for 30 - 90 minutes until reattached, or were collected for replating. The detached cells were collected by gentle pipetting in 199 + 10% NBCS, centrifuged at 1,000 rpm for five minutes, resuspended in a small volume of 199 + 10% NBCS and replated on fresh coverslips. Receptor activity was measured 30 - 90 minutes later. Appropriate controls were handled in parallel for each type of treatment. Ingestion and attachment of antibody coated sheep red blood cells (AbSRBC) were scored for 25 1:1 heterokaryons and 100 macrophages in duplicate preparations. Melanoma cells were always negative.

The following reagents were used.

Trypsin 2x crystalline, Worthington.

Soybean trypsin inhibitor (STI), Sigma Chemical Co. 2x crystalline.

Heat-inactivated trypsin, heated for 10 minutes at 100°C in H_2O.

Pronase, Calbiochem.

Chymotrypsin, Worthington.

Papain, Worthington, dissolved in 199 + 0.01 M cysteine HCl, pH 7.2.

β-glucosidase, Worthington 3.1 u/mg.

β-galactosidase, Worthington 320 u/mg.

Disodium Ethylenediamine tetraacetate (EDTA), Fisher Scientific Co., in PD.

Two neuraminidase preparations were used. C. vibrio enzyme, supplied by Behringwerke, through Certified Blood Products, N. Y., stated activity 500 u/ml, and a Cl perfringens enzyme obtained from Sigma Chemical Co., stated activity 1 u/mg.

Neuraminidase activity in medium 199 was compared with that in 0.1 M acetate buffer, pH 5.0, using bovine submaxillary mucin (Sigma) as substrate. Free sialic acid was determined by the procedure of Warren (1959).

4. The role of serum factors in the disappearance of heterokaryon Fc receptor activity was examined in the following way. Freshly harvested macrophages were cultivated in 199 + 15% fetal calf serum containing no IgG on immunoprecipitin assay, (IPT FCS, bought from Grand Island Biolog. Co., L. I.). Heterokaryons were prepared after 1 day. One hour after fusion, three groups of coverslips were incubated in either 199 + 15% IPT FCS, 199 + 1% 2x crystalline bovine serum albumin (BSA) or 199 + 1% fraction V bovine albumin. Receptor activity was assayed at 4 and 20 hours. Some preparations were treated

with 100 μg/ml trypsin for 30 minutes at 37°C.

In other experiments fused cells were incubated in non-specific bovine gamma globulin (BGG), starting one hour after fusion. Groups of coverslips were placed in medium 199 containing one of the following: 20% NBCS, 20% IPT-FCS, 20% IPT-FCS + 1 mg/ml BGG or 20% IPT-FCS + 10 mg/ml BGG. Receptor activity was assayed 2, 9 and 21 hours after fusion. Cells were washed 4x with 199 before the assay, to insure that no free BGG was still present. Crystalline BSA and Fraction V were obtained from Armour Pharmaceutical Co.; BGG from Pentex Chemical Co.

5. The Effect of Inhibitors of Protein and RNA Synthesis on Receptor Disappearance. Groups of coverslips were treated with cycloheximide (5 μg/ml), starting one hour after fusion. Treatment was continued for four - eight hours, depending on the particular experiment. Cells were washed 3x in 199 to stop treatment. Fc receptor activity was assayed immediately after treatment, or at a later time.

Bromotubercidin treatment, 5 μg/ml, was either started in cocultivated cells some time before fusion, or after fusion. When necessary, bromotubercidin was present during fusion as well as subsequently, to maintain continuous treatment.

6. Ultra-violet irradiation of cells before fusion. Macrophages or melanoma cells were cultivated in Falcon 60 mm dishes for one day. The cells were washed 2x with 199, covered with 1 ml 199 and irradiated with a Sylvania germicidal lamp (G15T8) for 10 - 90 sec at a distance of 20 cm. These conditions proved suitable for subsequent studies. After irradiation the cells were washed and incubated in 199 + 10% NBCS. Cells were fused one to two hours after ultra-violet

treatment and incubated in 199 + 20% NBCS until ready for assay of receptor activity.

7. Fusion of Macrophages with Other Cells. Chick embryo red blood cells were obtained from 12 day chick embryos as described in Chapter III.

Chick primary fibroblast cultures were prepared from chick embryos using standard procedures (Wasley and May, 1970).

Ehrlich ascites tumor (E. A. T.) cells were obtained from Dr. E. Borenfreund, Sloan-Kettering Institute. These cells were passaged serially in the peritoneal cavity of NCS mice and harvested four to five days after inoculation.

Macrophage monolayers were prepared on coverslips for fusion. 1×10^4 to 1×10^6 of the above cells were inoculated per coverslip and fused one hour later, using 1,000 hemagglutinating units of ultraviolet-inactivated Sendai virus. After 30 - 60 minutes the preparations were washed and incubated further in 199 + 20% NBCS. Preparations were assayed for the Fc receptor at intervals after fusion.

Many chick embryo red cells and most E. A. T. cells which had not fused with macrophages were removed by washing after fusion. The macrophages ingested the remaining unfused red blood cells so that by one day pure populations of macrophages and fused cells remained on the coverslips. The E. A. T. cells did not stick to glass, nor could they grow in tissue culture, so that only those E. A. T. cell nuclei which were "trapped" by fusion with the macrophages survived in culture. All these heterokaryons were identified on the basis of nuclear morphology.

8. The effect of the antiserum concentration used to coat SRBC, on Fc receptor activity was studied as follows. A 1% suspension of

SRBC was incubated with different concentrations of rabbit antiserum (1/12, 500 - 1/1, 000), using the procedure described above. The coated cells were washed 2x in 10 ml saline and then resuspended in 199 at a final concentration of 0.1% . Fused preparations were assayed in duplicate. The mean number of attached (A) and ingested (I) red cells was determined for fused, as well as unfused cells. The ratio $\frac{I}{A+I}$ was defined as the ingestion index.

The relationship between protein synthesis and the ingestion index of newly fused cells was studied with the use of cycloheximide. Cocultivated monolayers of macrophages and melanoma cells were prepared. Cycloheximide (5 μg/ml 199 + 20% NBCS) was added fifteen minutes before fusion and was continually present up to three hours after fusion. Fc receptor activity was determined in the presence of cycloheximide as well.

Results

1. Phagocytosis. The ability of the macrophage to ingest particles coated with 7S antibody is an example of a specific cell function. Such particles become attached to specific receptors at the cell surface and their ingestion follows rapidly under the appropriate conditions.

Sheep red cells were coated with rabbit antiserum and their uptake by fused cells examined. Experiments assaying phagocytic function are illustrated in Figs. 15 - 17 and the results of a typical experiment are listed in Table XI. In macrophage homokaryons there is a direct relationship between the number of macrophage nuclei present and the mean number of red cells ingested per cell (Fig. 16). The phagocytic activity of heterokaryons at different times after fusion is illustrated in Fig. 17. Each type of heterokaryon was compared with homokaryons containing the same number of macrophage nuclei (Table XI).

Figure 15 (a-e)

The phagocytosis of opsonized sheep erythrocytes by homokaryons, heterokaryons and unfused macrophages. Stained x 1000.

Fig. 15a. An unfused macrophage has ingested many erythrocytes whereas an adjacent melanoma cell contains none.

Fig. 15b. Extensive red cell phagocytosis by a multinucleated homokaryon.

Fig. 15c. A 2:1 heterokaryon two hours after fusion. The macrophage nuclei have already enlarged and the cell has phagocytized many erythrocytes.

Fig. 15d. A 2:1 heterokaryon twelve hours after fusion. Only two erythrocytes have been ingested. Adjacent, unfused macrophages with smaller nuclei contain many erythrocytes.

Fig. 15e. A 2:1 heterokaryon has failed to ingest erythrocytes 24 hours after fusion.

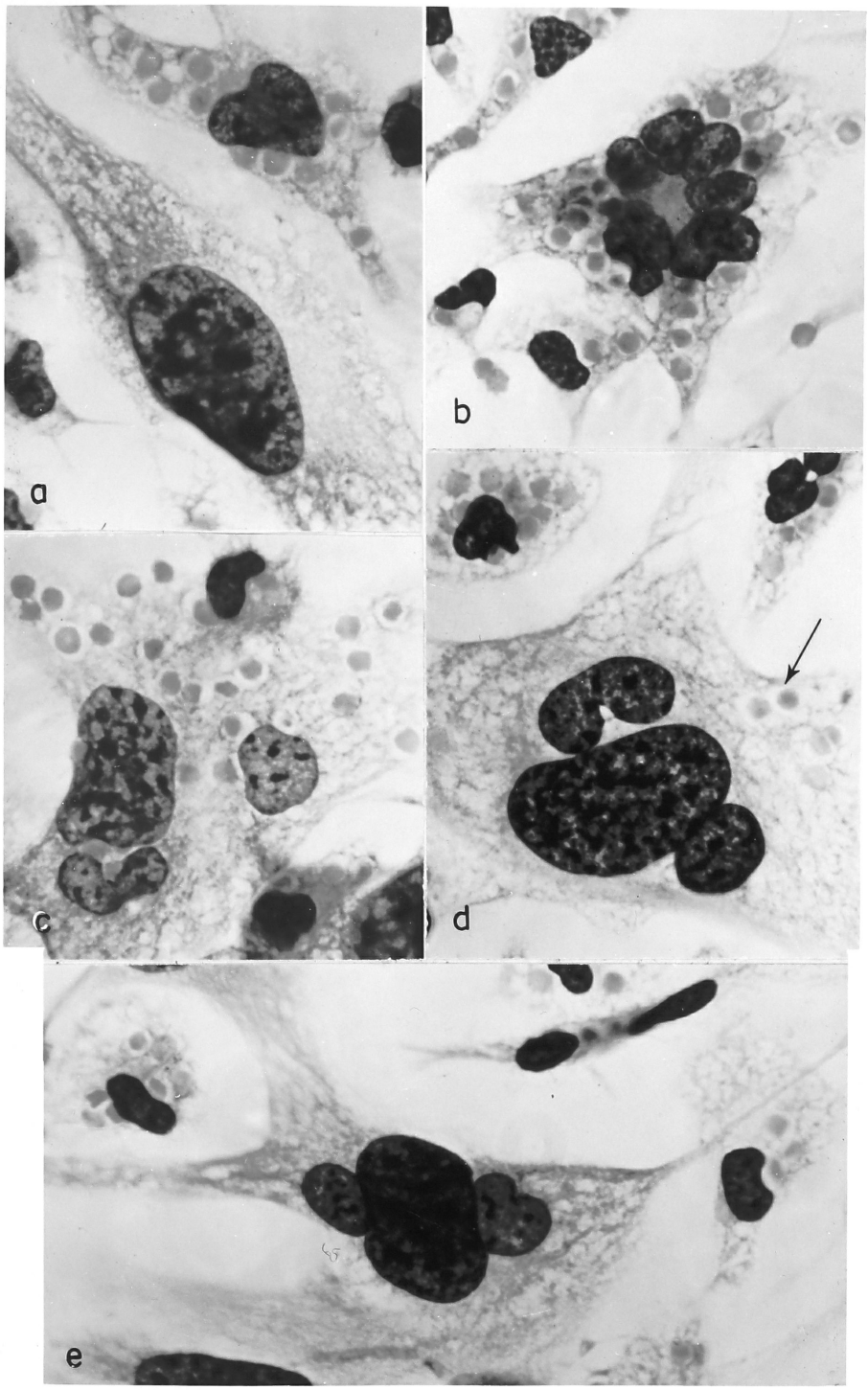

a
b
c
d
e

TABLE XI

Phagocytosis by macrophage homokaryons and Macrophage-melanoma cell heterokaryons

Time after fusion	Type of cell (mac:mel)	Number of cells scored	Mean no. rbc ingested per cell	Std. deviation of the mean	% of control*
4 hrs.	1:0	30	2.8	.31	
	2:0	25	7.3	.57	
	3:0	11	9.9	1.3	
	1:1	39	2.5	.52	89
	2:1	18	6.7	1.2	92
	3:1	9	8.9	1.6	90
9 hrs.	1:0	31	3.5	.33	
	2:0	30	6.8	.65	
	3:0	22	9.6	1.2	
	1:1	52	1.8	.44	51
	2:1	23	5.1	1.1	75
	3:1	13	10.4	2.5	100
1 day	1:0	30	3.1	.36	
	2:0	26	4.1	.45	
	3:0	25	7.6	.59	
	1:1	81	0.65	.098	21
	2:1	34	2.3	.53	56
	3:1	16	5.2	.67	69
2 days	1:0	10	10	1.2	
	2:0	12	12.7	1.1	
	3:0	11	22	2.8	
	1:1	113	1.3	.24	13
	2:1	31	3.3	.76	26
	3:1	12	8.5	1.8	39
4 days	1:0	40	7.5	.62	
	2:0	43	8.7	.71	
	3:0	40	11.1	.96	
	4:0	30	15.1	1.5	
	1:1	68	0.29	.089	3.8
	2:1	46	0.91	.34	11
	3:1	9	1.7	.76	15
5 days	1:0	10	6.4	1.0	
	2:0	10	9.4	.95	
	3:0	10	16.7	2.2	
	1:1	44	0.34	.13	5.3
	2:1	22	0.82	.35	8.8
	3:1	13	0.70	.26	4.2

*Each type of heterokaryon compared with homokaryon containing same number of macrophage nuclei.

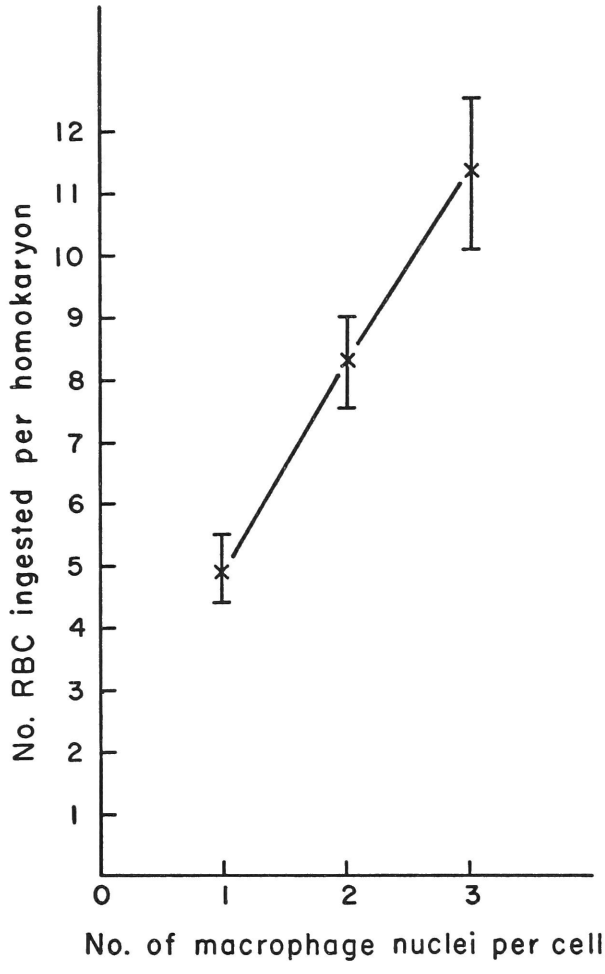


Figure 16. The ingestion of sensitised erythrocytes by macrophages and macrophage homokaryons. The crosses represent the mean number of red cells ingested, the bars 2 x the standard deviation of the mean.

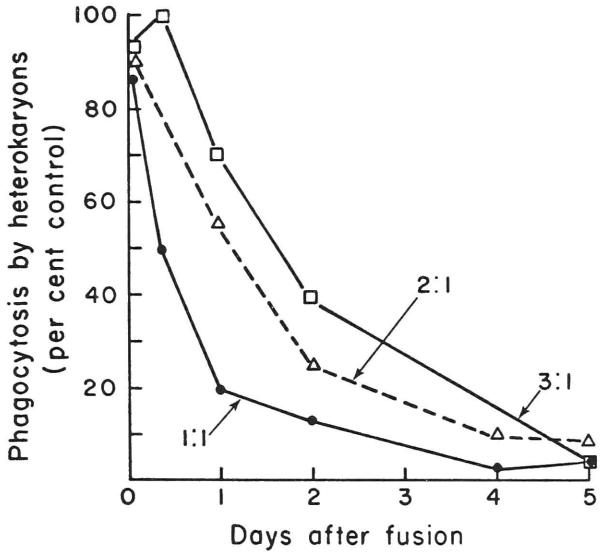


Figure 17. Phagocytosis by macrophage-melanoma cell heterokaryons. Macrophage homokaryons containing the same number of macrophage nuclei served as controls.

Heterokaryons studied soon after fusion ingested as many red cells as their controls, but this ability disappeared within a few days (Fig. 15). The number of macrophage nuclei present in a heterokaryon influenced the loss of phagocytic function.

The initial rate of loss of phagocytic function in 1:1 heterokaryons is illustrated in Fig. 18. It can be seen that the decay curve is exponential, with a half-life of 2.7 hours.

To determine whether the failure of older heterokaryons to ingest red cells was due to a failure in attachment, heterokaryons of different ages were exposed to the red cells for fifteen minutes at room temperature, washed 4 x and fixed immediately. Whereas almost all macrophages had three or more attached red cells, none were present on the melanoma cells. In heterokaryons the attachment of antibody coated red cells again decreased with time, at a rate which depended on the number of macrophage nuclei present in the heterokaryon. The failure to ingest red cells is therefore associated with a failure in attachment. Unless noted to the contrary, this also applies to all subsequent experiments.

As shown later (Table XXI) heterokaryons require higher concentrations of opsonizing antiserum to bind and ingest AbSRBC than macrophages. The experiments in this section employed sufficiently high serum concentrations (1/2000 or 1/3000) to make this effect negligible.

To gain further insight into the mechanism of disappearance of the macrophage surface marker heterokaryons were cultivated in medium containing high or low serum concentration (Fig. 19). The results show that the loss of phagocytic function in heterokaryons was accelerated in the presence of the higher concentration of calf serum. To determine if phagocytic function reappeared at later times heterokaryons were prepared by the trypsinization procedure and followed

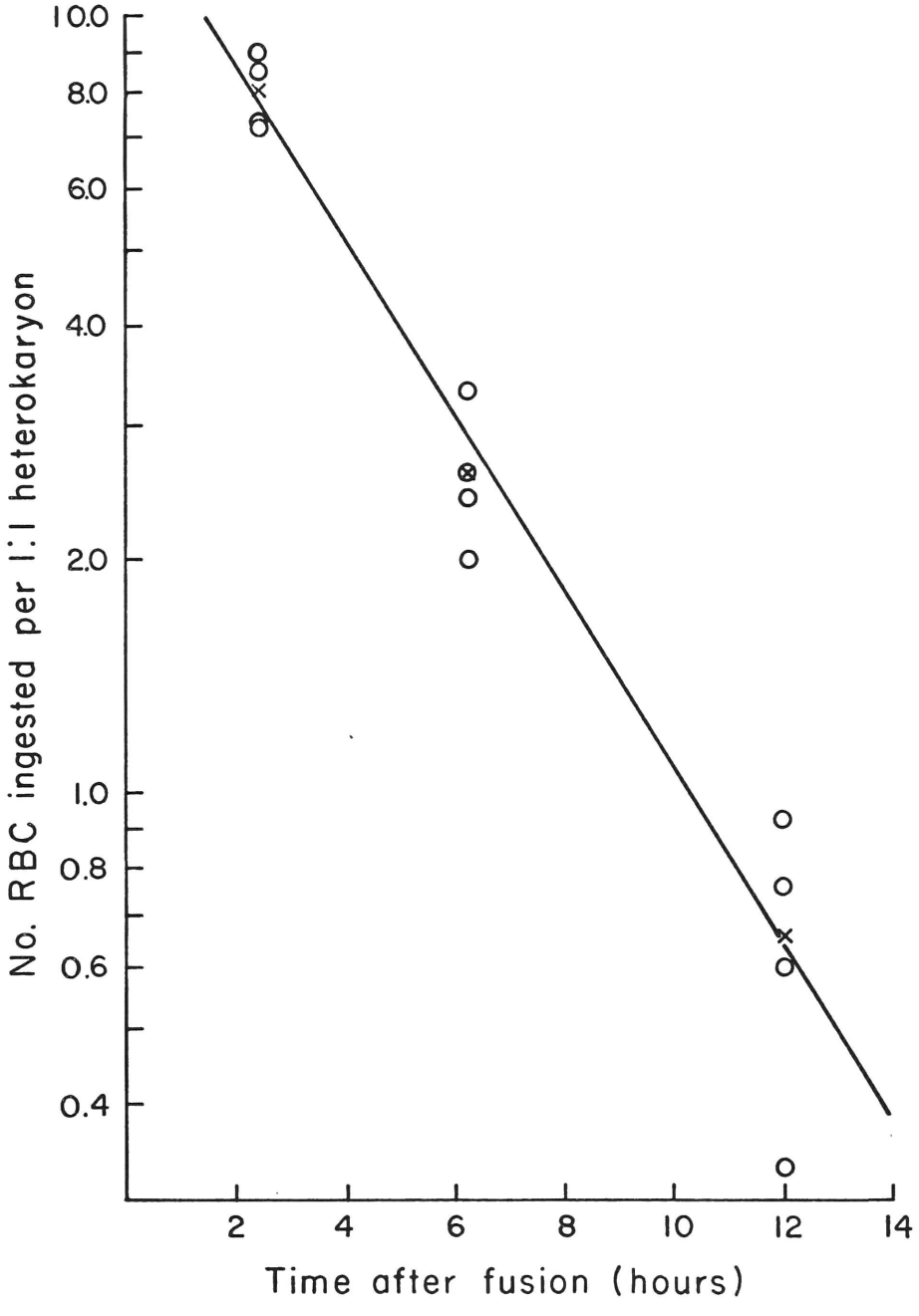


Figure 18. The early loss of phagocytic function in 1:1 heterokaryons. The circles represent the mean number of RBC ingested by 30 heterokaryons on a particular coverslip, the crosses represent the mean for each group of four coverslips.

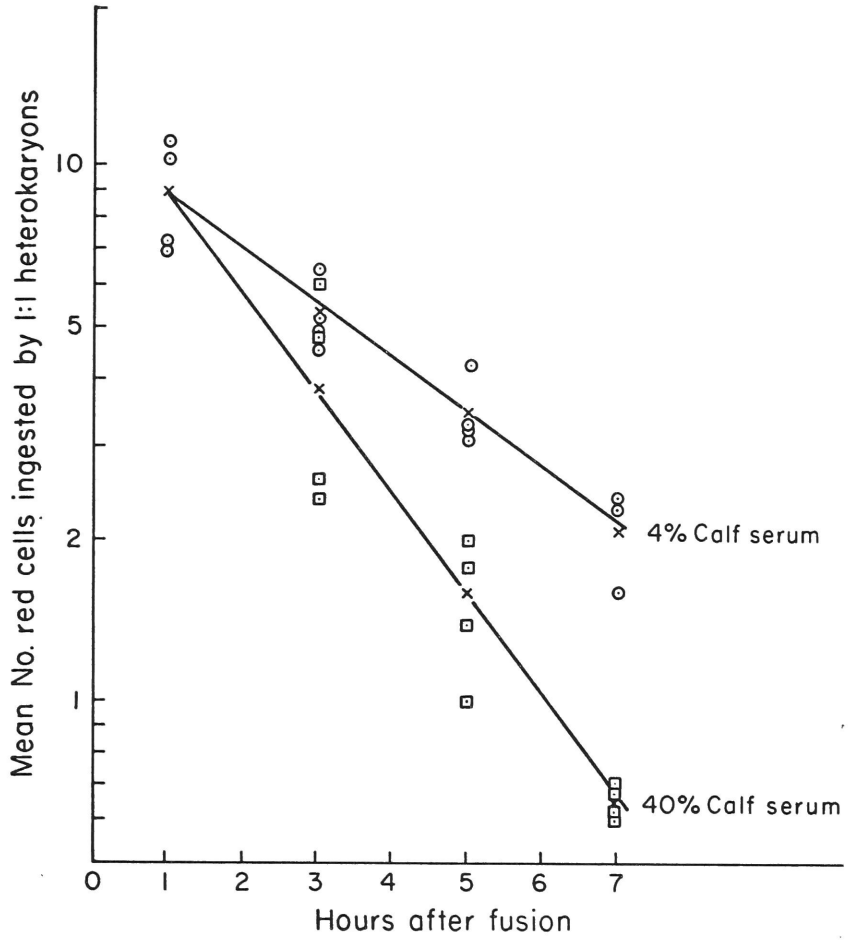


Figure 19. The effect of serum concentration on red cell uptake by 1:1 heterokaryons. Starting one hour after fusion, one group of coverslips cultivated in 199 + 4% NBCS, the other in 199 + 40% NBCS. The circles and squares represent the mean number of RBC ingested by 30 heterokaryons per coverslip, the crosses the mean for each group of four coverslips.

for one week. The loss of phagocytic function from 1:1 heterokaryons was almost complete by the second day after fusion and did not return by the seventh day. These heterokaryons have very prominent macrophage nucleoli from the first day after fusion.

The fate of the phagocytic marker in heterokaryon progeny was studied by using the dextran sulfate label and the trypsinization method to prepare 1:1 heterokaryons (Chapter II). The loss of phagocytic ability was virtually complete before the first division and no daughter cells, which were identified by the presence of dextran sulfate, recovered this property over the next week.

2. ATPase Activity. Intact macrophages have thirty times more surface ATPase activity than melanoma cells and this difference is sufficient to be useful as a cytochemical marker. It was possible to follow the presence as well as the distribution of this macrophage surface marker at various times after the formation of heterokaryons.

Preliminary experiments were performed to determine some of the properties of the enzyme under study. The substrate specificity of the enzyme is shown in Table XII. The enzyme is specific for nucleoside 5' phosphates and ATP is hydrolysed most actively. The rather active hydrolysis of ADP and, to a lesser extent AMP (5'), suggests that stepwise P_i release could be occurring during the incubation procedure. The enzyme showed an absolute requirement for Mg^{++}, which could be almost completely replaced by Ca^{++}. Neither Na^{+} nor K^{+}, nor both ions together were in fact necessary for maximal enzyme activity and ouabain had no effect on the total activity.

The cytochemical procedure stained the surface of macrophages strongly and diffusely whereas melanoma cells showed no reaction product under the light microscope. Some peripheral vesicles,

TABLE XII

The liberation of Pi from different substrates by macrophage surface ATPase

Substrate	μm Pi/μg protein/min	percent activity (ATP = 100)
ATP (sodium salt)	3.0×10^{-2}	100%
ITP	2.3×10^{-2}	77%
ADP	1.9×10^{-2}	63%
GTP	1.3×10^{-2}	43%
CTP	1.1×10^{-2}	37%
AMP (5')	0.81×10^{-2}	27%
AMP (2', 3')	0	0
dAMP (5')	0	0
Cyclic AMP	0	0
Glucose-6-Phosphate	0	0
Na α-naphthyl acid Phosphate	0	0
Disodium β-glycerophosphate	0	0

thought to be derived from the cell surface, were also stained. The reaction product was seen either as a margin close to the cell periphery or as a granular deposit over the cell surface. Electron microscopic cytochemistry confirmed the surface distribution of the reaction product and the absence of intracellular staining.

The fate of macrophage ATPase in heterokaryons is shown in Fig. 20. It is clear that the original ATPase introduced by the macrophage disappears from heterokaryons in a macrophage dose related fashion.

The distribution of reaction product in heterokaryons was of interest and is illustrated in Fig. 21. In the first twelve hours after fusion, when heterokaryons were still staining, the reaction product occasionally involved only a "patch" of the total cell surface. Many heterokaryons, including 1:1 cells, showed either a faint, diffuse surface distribution or a margin about the cell. Patches were still seen in a few 3:1 heterokaryons after one day, but the reaction product was usually faint and diffuse by thirty-six hours.

3. Mechanisms Which Could Account for Membrane Changes in Heterokaryons. These studies of the macrophage membrane markers in fused cells revealed two phenomena. The Fc receptor functioned in the heterokaryons soon after fusion, but phagocytic activity then became undetectable, whereas macrophage homokaryons continued to express this property. The ATPase activity also disappeared from heterokaryons and in addition seemed to spread out over the cell surface. Several possible mechanisms, schematically drawn in Fig. 22, could account for these findings.

1. Dilution. As the receptor spreads out it could cease to function, if a critical receptor density is required to bind antibody coated red cells, for instance.

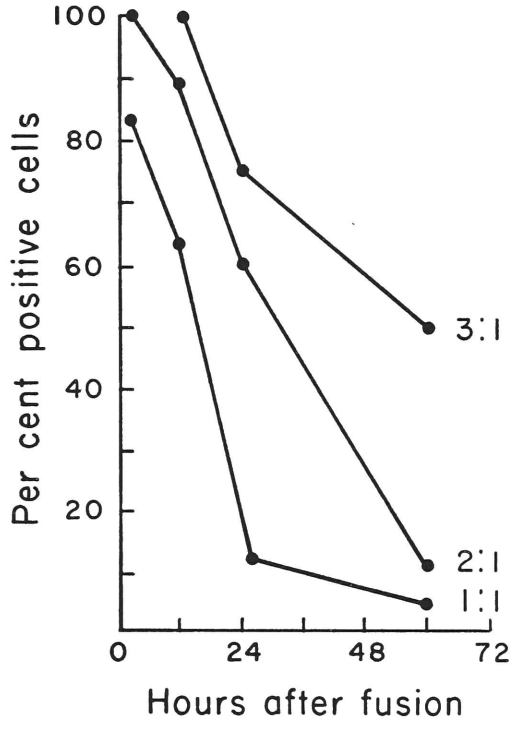


Figure 20. The loss of cytochemically demonstrable ATPase activity in heterokaryons.

Figure 21 (a-d)

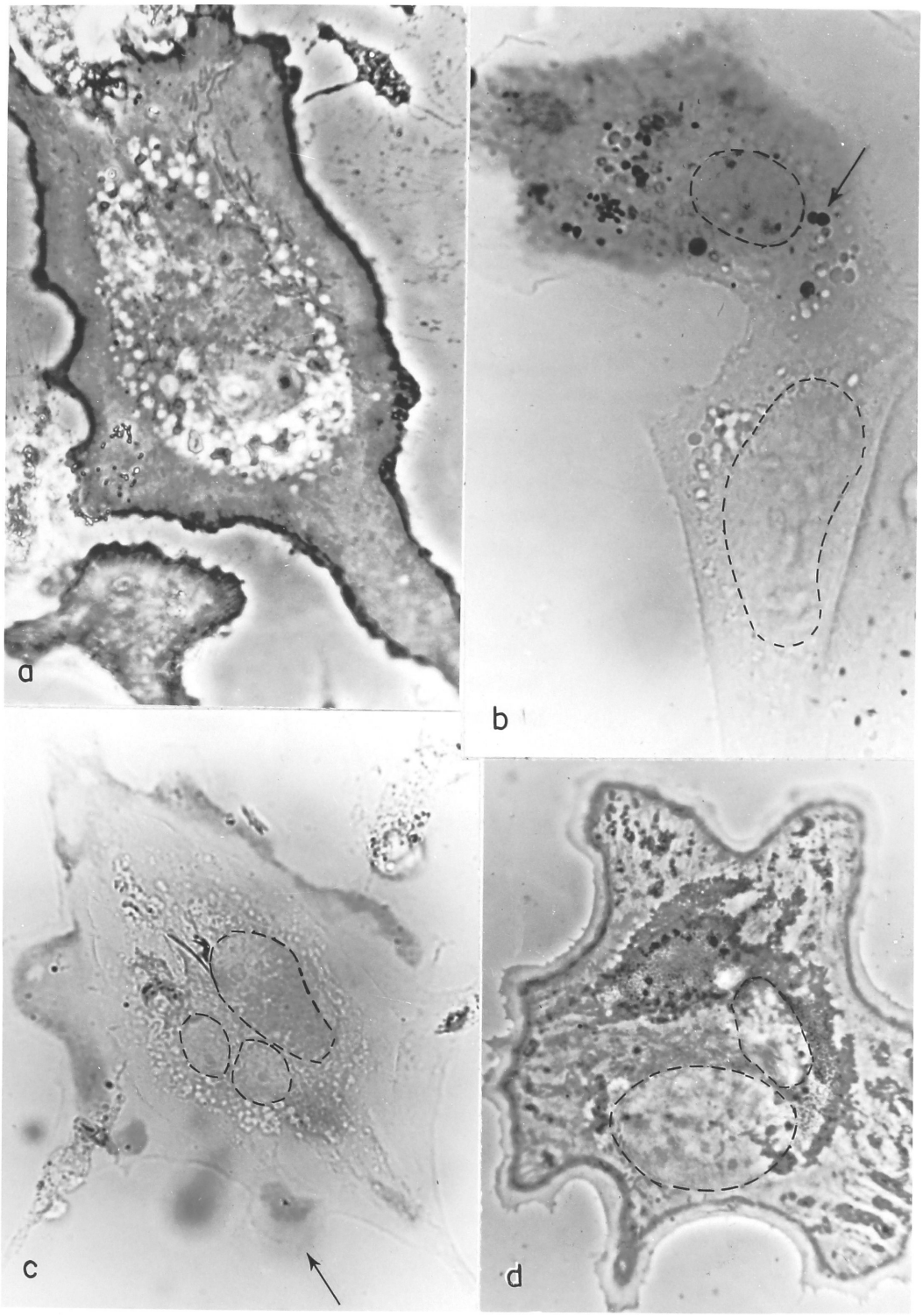
The cytochemical demonstration of ATPase activity in homokaryons and heterokaryons. Fixed in 1. 25% glutaraldehyde prior to incubation with substrate.

Fig. 21a. A macrophage homokaryon exhibiting intense reaction product about the cell periphery. Phase contrast. x 1000.

Fig. 21b. An early stage in the formation of a 1:1 heterokaryon. The upper macrophage shows reaction product diffusely distributed over its surface. A number of intracellular vesicles (arrows) are also stained. In contrast, the melanoma cell surface and vesicles are free of reaction product. Bright field. x 1000.

Fig. 21c. A 2:1 heterokaryon three hours after fusion. Reorganization of the nuclei and cytoplasm have occurred but distinct patches of reaction product are present on the cell surface. One isolated patch is marked with an arrow. Bright field. x 800.

Fig. 21d. A 1:1 heterokaryon eight hours after fusion. A fine reaction product outlines the cell periphery. Coarser aggregates are seen over the cell surface. Bright field. x 1200.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Possible mechanisms for "loss" of Fc receptor

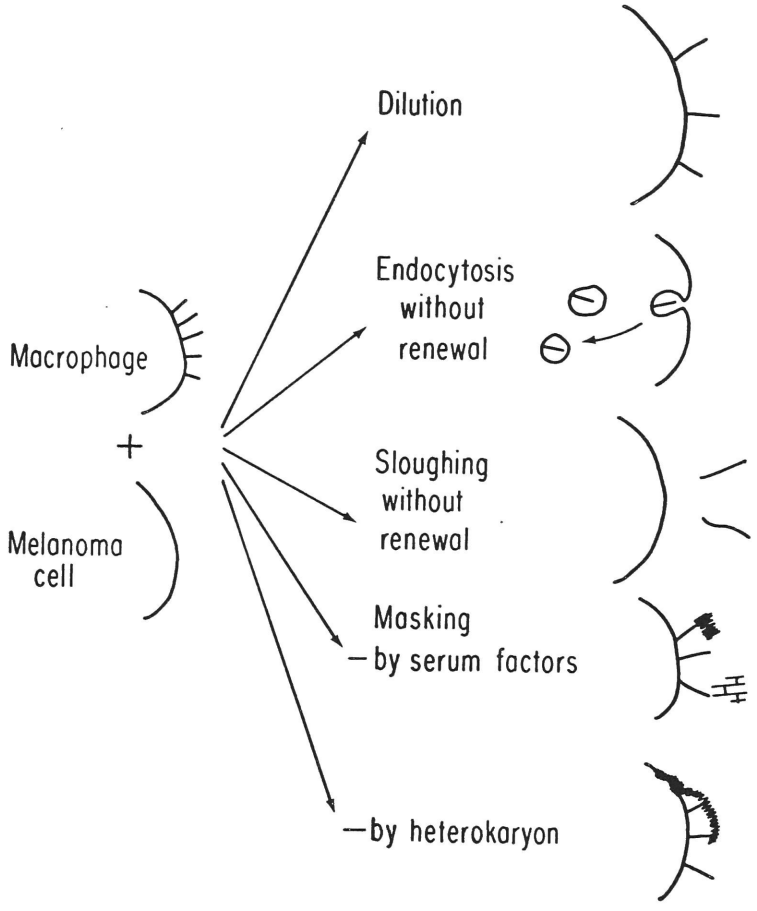


Figure 22

2. Endocytosis without renewal. The membrane receptor could flow into the cell during ongoing pinocytosis and fail to be resynthesized in the heterokaryon.

3. Sloughing without renewal. The receptor could be lost into the incubation medium and not be renewed.

4. Masking. a. Material from the serum, used for cultivation, could block the receptors. This could be γG antibody or perhaps other substances.

b. Finally -- the heterokaryon could itself produce such masking substances which either blocked the receptor through a direct steric mechanism or which altered membrane conformation in a more subtle fashion.

Further experiments showed that the last hypothesis (4b) could explain the apparent loss of the Fc receptor.

4. The Recovery of the Phagocytic Receptor in Heterokaryons. If 1:1 heterokaryons which have lost their Fc receptor activity are treated with trypsin, they recover all the phagocytic function initially demonstrable after fusion (Figs. 23, 26a). Upon further incubation in the absence of trypsin the receptor again becomes undetectable. Table XIII illustrates the effect of trypsin concentration on receptor activity. Receptor activity is fully active after treatment with 1 - 10 μg/ml trypsin (30', 37°C). The subsequent decline in Fc activity also depends on the dose of trypsin used. These changes occur in heterokaryons without any effect on the function of unfused macrophages.

The effect of trypsin is not an artifact due to selection of a macrophage-rich heterokaryon population. Table XIV illustrates an experiment in which trypsin treatment was limited to brief periods at room temperature. Receptor activity was recovered after six minutes

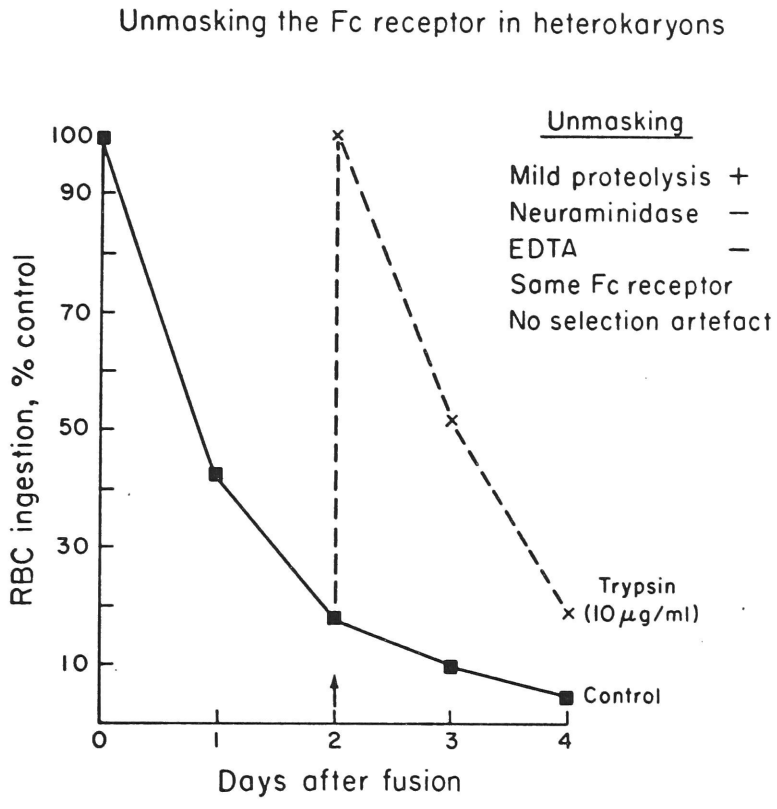


Figure 23. Recovery of the phagocytic receptor in heterokaryons

TABLE XIII

The effect of trypsin treatment on the ingestion of antibody coated SRBC

Treatment (μg/ml Trypsin)	Time after Treatment Hours	1:1 heterokaryons	Macrophages	Ratio $\frac{\text{Heterokaryon}}{\text{Macrophage}}$ x 100
Control	-24	3.2	6.6	50
		2.7	6.6	41
	0.5	2.4	8.2	29
		1.2	7.1	17
	3	1.6	6.7	24
	24	1.1	6.3	17
		1.0	5.7	18
	48	.55	9.3	5
10	0.5	5.4	5.3	102
		5.9	6.6	90
	3	5.8	5.6	104
	24	2.2	4.8	46
		3.8	6.5	58
	48	.80	6.0	13
		.36	5.4	7
1	0.5	6.1	6.6	93
		4.9	6.5	76
	3	5.0	7.0	70
		4.5	6.1	75
	24	3.5	6.5	54
		2.8	6.8	41
	48	.50	7.0	7
0.1	0.5	3.2	6.0	53
		2.1	6.1	34
	3	2.3	6.8	34
	24	2.0	6.8	30
		1.8	7.4	24

Trypsin treatment, two days after fusion, for 30' at 37°C. Incubated in 199 + 15% NBCS after treatment. SRBC coated with 1/2,000 antiserum. Assayed in situ, in duplicate.

TABLE XIV

In situ unmasking of 1:1 heterokaryons after brief trypsin treatment

Concentration µg/ml	Duration of Treatment Mins.	Mean no. AbSRBC ingested
5	2	.2
	4	.9
	6	3.3
	8	6.3
2	2	.8
	4	.8
	6	4.4
	8	3.2

Trypsin treatment, 2 days after fusion, at room temperature. Washed 3x and assayed immediately.

treatment with 2 μg/ml trypsin when no heterokaryons had yet become detached from glass. In different experiments all heterokaryons were detached from coverslips by treatment with 100 μg/ml trypsin for 30 minutes at 37°C and replated on a fresh coverslip. Full receptor activity was again recovered.

Trypsin treatment does not alter the specificity of the interaction between antibody coated red cells and the Fc receptor. Melanoma cells still do not bind any AbSRBC and the presence of antibody on the red cell is obligatory for both attachment and ingestion.

Further experiments, in which a variety of enzymatic and chemical treatments were employed, are summarised in Table XV. Active proteolysis is required for the recovery of receptor activity. Heat inactivated trypsin or the presence of soybean trypsin inhibitor prevented this effect. Other proteolytic enzymes like pronase, chymotrypsin and papain are equally effective. EDTA was ineffective at a concentration which detached heterokaryons from the glass. Such EDTA treated heterokaryons rapidly reattached to the glass in fresh 199 + 20% NBCS.

Neuraminidase treatment was also ineffective. Control experiments confirmed that the enzyme when used in medium 199 retained 77% of its activity in acetate buffer at pH 5.0.

These findings gave rise to the hypothesis that proteolytic treatment probably removed protein(s), from the cell surface, which had masked receptor activity and that on subsequent cultivation, the receptor once again became masked.

5. The Role of Serum Factors in Blocking the Heterokaryon Fc Receptor. Masking proteins could be adsorbed from the serum in the culture medium or synthesized by the heterokaryon itself. Adsorbed proteins could be nonspecific gamma globulins or other serum proteins.

TABLE XV

The effect of chemical and enzymatic treatments on masked heterokaryons*

Treatment	Ingestion of AbSRBC 1:1 Heterokaryons**	Macrophages***	Ratio $\frac{\text{Heterokaryons}}{\text{Macrophages}}$ x 100
Nil	.60	6.3	10
Trypsin (5 μg/ml)	4.3	5.5	78
" heat inactivated	.78	7.8	10
" +12 μg/ml STI	.52	9.6	5
" + 6 μg/ml STI	1.3	7.0	19
Pronase (10 μg/ml)	9.8	8.8	111
Chymotrypsin (10 μg/ml)	5.3	6.5	82
Papain - control (199+ .01M cysteine HCl)	1.5	6.5	23
Papain (10 μg/ml) (in 199+ .01M cysteine HCl)	6.0	6.1	99
EDTA control (Ca, Mg free buffer)	.95	6.0	18
EDTA 5.4×10^{-4} M	1.2 1.1	9.9 7.3	12 11
Neuraminidase -C. Vibrio (100 u/ml)	1.8	7.9	23
Cl perfringens (1 u/ml)	1.1	N.D.	--
β-Galactosidase (10 μg/ml)	.85	6.2	14
β-Glucosidase (500 μg/ml)	.80	7.2	11

*Fused preparations cultivated in 199 + 20% NBCS for 1 day, washed 2x and treated 30'/37°C with appropriate reagent. Assayed, in situ, 0 - 90 mins. after treatment.

**Mean for 25 heterokaryons.

***Mean for 100 macrophages.

Cells were therefore cultivated in the absence of gamma globulin or placed in a medium in which BSA was the only protein. In all cases (Table XVIA) receptor activity dropped to 15 - 30% of the initial activity in 20 hours. This effect was reversed by treatment with trypsin.

In other experiments nonspecific BGG was added to the culture medium after fusion (Table XVIB). This experiment showed that the rate of "disappearance" of the Fc receptor could be slightly accelerated by adding large quantities of BGG (10 mg/ml) to the medium, but was not a prerequisite for its loss.

It was therefore concluded that serum proteins were not the major cause of the blocked receptors.

6. The Effect of Inhibitors on Receptor Disappearance. If the heterokaryon made proteins which blocked the Fc receptor, could this process be prevented?

An experiment in which cycloheximide was used to block protein synthesis after fusion is illustrated in Fig. 24. Unlike the control preparation, in which receptor activity was lost, the cycloheximide treated heterokaryons maintained full phagocytic activity. Receptor activity in the unfused macrophages was unaffected by cycloheximide treatment. When the block in protein synthesis was reversed by washing out the drug, receptor activity was lost subsequently and could again be uncovered with trypsin.

In another experiment cells were treated with cycloheximide for different times and the reversible inhibition of masking studied in more detail (Table XVII). Receptor activity could be maintained for eight hours after cycloheximide treatment. Removal of the drug after four or six hours resulted in loss of receptor activity at a rate similar to that of the untreated control. The cycloheximide effect was still

TABLE XVI

A. The masking reaction in the absence of serum factors

Medium	4 hrs after fusion		Uptake of AbSRBC by 1:1 Heterokaryons 20 hrs after fusion			
			No trypsin		Trypsin	
	Mean	S. E.	Mean	S. E.	Mean	S. E.
199+15% IPT FCS	13	1.5	3.0	.57	12	2.0
199+1% Fraction V	15	1.5	2.1	.54	16	3.3
199+1% BSA	14	1.8	4.3	.90	13	2.0

B. The effect of incubation in nonspecific BGG on the ingestion of AbSRBC by 1:1 heterokaryons

Time after fusion	NBCS Control	IPT-FCS	IPT-FCS +1 mg/ml BGG	IPT-FCS +10 mg/ml BGG
2 hrs.	7.9	--	--	--
9 hrs.	1.8	4.0	3.5	1.5
21 hrs.	.64	1.4	.75	--

NBCS = newborn calf serum.

IPT-FCS = fetal calf serum without gamma globulin.

BGG = nonspecific bovine gamma globulin.

BSA = bovine serum albumin.

The requirement for protein synthesis in masking

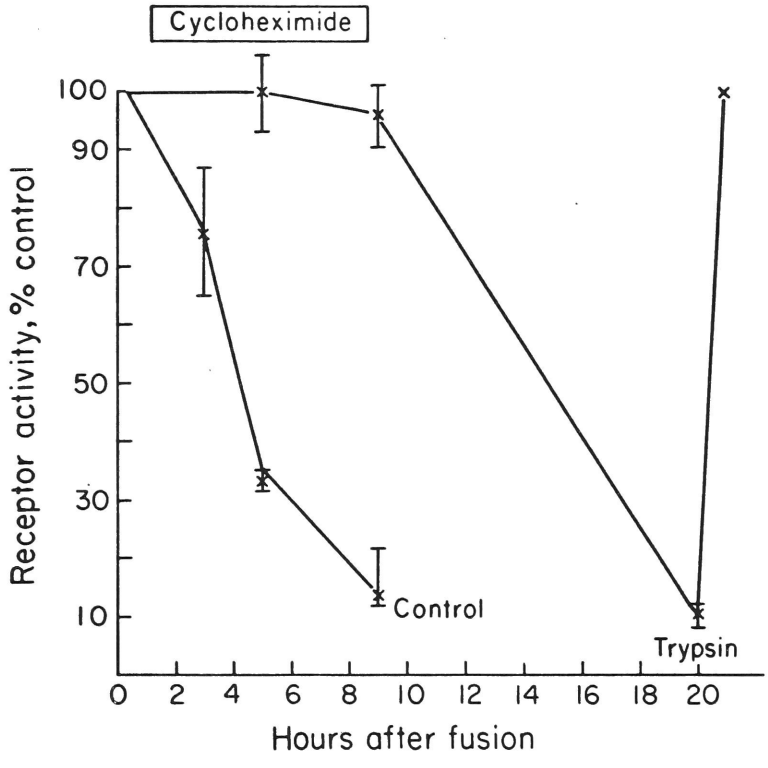


Figure 24

TABLE XVII

The reversible inhibition of masking by cycloheximide*

	Mean no. AbSRBC ingested by 1:1 heterokaryons			
Hours after fusion	Control	CH 1-5**	CH 1-7	CH 1-9
1	5.6	--	--	--
5	3.4	5.0	--	--
7	3.0	3.4	6.3	--
9	2.1	1.7	4.2	6.4
11	0.9	1.3	2.5	7.6
22	--	--	--	2.2

*Groups of coverslips treated with 5 μg/ml cycloheximide for 4, 6 or 8 hours starting 1 hr after fusion. The cells were then washed 3x, incubated in 199 + 20% NBCS and assayed at different times.

**Cycloheximide treatment 1 - 5 hrs after fusion.

reversible eight hours after treatment but with a two hour lag period.

Some unfused macrophages are killed by six to eight hours continuous exposure to cycloheximide, but heterokaryons and melanoma cells are able to survive. Even an eight hour treatment with cycloheximide does not interfere with phagocytosis in active heterokaryons (Fig. 26b) or surviving macrophages.

Cells were also treated with cycloheximide for six to eight hours, starting 20 hours after fusion, to see if the receptor could be recovered in this way. Receptor activity remained masked in this experiment. This meant that cycloheximide treatment, per se, did not bring about increased receptor activity in the heterokaryons, but that protein synthesis had to be blocked during the period immediately after fusion.

The role of RNA synthesis in masking the Fc receptor was investigated next, using bromotubercidin as a reversible inhibitor (Table XVIII). When heterokaryons were treated with bromotubercidin one to nine hours after fusion there was no effect on masking, whereas cycloheximide treatment during the same period prevented masking (Table XVIIIa). If, however, the bromotubercidin treatment was started two hours before fusion, masking was effectively prevented (XVIIIb). This inhibition of masking was reversible even after twelve hours treatment (XVIIIc).

7. Ultra-violet Irradiation of Cells Before Fusion. Melanoma cells and macrophages were irradiated with ultra-violet light before fusion to separate the contribution of each heterokaryon nucleus to the masking reaction. The effect of such treatment is shown in Table XIX. Melanoma cell pretreatment preserved phagocytic function in 1:1 heterokaryons, whereas macrophage pretreatment had no effect on receptor loss.

Both cell fusion and phagocytic activity were unaffected by ultra-

TABLE XVIII

The requirement for RNA synthesis to mask the Fc receptor in 1:1 heterokaryons

A. Bromotubercidin (BT) treatment 1-9 hrs after fusion

Hours after fusion	Ingestion of AbSRBC		
	Control	Cycloheximide*	Bromotubercidin treatment
2.5	9.0	--	--
5	3.2	10.2	4.7
9	1.7	8.1	2.8

B. Continuous Bromotubercidin treatment started before, or after, fusion

Hours after fusion	Ingestion of AbSRBC			
	Control	BT started 2 hrs after fusion	BT started 0.5 hr after fusion	BT started 2 hrs before fusion
2	7.0	--	--	9.7
7	1.8	2.8	3.8	8.2

C. The reversible inhibition of masking by Bromotubercidin treatment

Hours after fusion	Ingestion of AbSRBC		
	Control		Bromotubercidin treatment (started 5 hrs before fusion)
2	5.8		5.1
5	3.0		6.4
7	2.4	Washout BT	7.4
23	1.0		1.5

*Cycloheximide treatment 1-9 hrs after fusion.

TABLE XIX

The effect of ultraviolet irradiation, before fusion, on the ingestion of AbSRBC by heterokaryons*

Fusion	Hours after fusion	1:1 Heterokaryons		Ratio $\frac{\text{Heterokaryons}}{\text{Macrophages}}$
		Mean	S. E.	
Control	3	10.5	1.3	1.05
	9	6.6	1.6	.67
	21	1.8	.62	.20
Ultraviolet Rx Melanoma cells	3	11.0	1.0	1.3
	9	9.5**	.71	1.3
	21	7.3***	.74	.82
Ultraviolet Rx Macrophages	3	9.9	1.0	1.0
	9	5.0	1.0	.65
	21	1.8	.36	.23

*Cells were irradiated for 10 secs at 20 cm distance.

**$t = 1.737$, $P < .05$.

***$t = 3.6066$, $P < .0025$.

violet pretreatment. Similar results were obtained after pretreating the cells for longer periods (30 or 90 sec.), although many irradiated cells were then dead one day later.

8. The role of fusion partner in the masking reaction was studied to determine if this process was unique to melanoma cell fusion. As shown in Table XX, fusion with Ehrlich ascites tumor cells, another malignant cell line, caused heterokaryon receptor activity to disappear even more rapidly. Macrophage-chick erythrocyte heterokaryons maintained receptor activity for seven days. After fusion with primary chick fibroblasts an intermediate result was obtained and receptor activity diminished more slowly.

It was concluded that fusion with rapidly growing cells, like tumor cells, resulted in rapid loss of the Fc receptor, whereas inactive nuclei, like those of the chick erythrocyte, could not bring about such an effect.

9. The Role of Antiserum Concentration in Fc Receptor Activity. In order to learn more about the properties of the macrophage receptor in heterokaryons we examined the uptake of sheep red blood cells coated with different concentrations of antiserum (Table XXI). In the absence of antiserum no red cells bind to macrophages. Increasing antiserum concentration first stimulates attachment and then triggers ingestion (Table XXIA). There is a striking difference between macrophages and three hour 1:1 heterokaryons in regard to the antiserum concentration at which ingestion is triggered. Apparently 1:1 heterokaryons require more antiserum than macrophages to ingest red cells and 2:1 heterokaryons behave in an intermediate fashion.

The role of protein synthesis in bringing about this difference between macrophages and heterokaryons was studied by maintaining cells

TABLE XX

The role of fusion partner on the ingestion of AbSRBC by 1:1 macrophage heterokaryons

Type of Heterokaryon x Macrophage	Time after fusion (Hours)	Ratio $\frac{\text{Heterokaryon}}{\text{Macrophage}} \times 100$
Melanoma cell	20	15
Ehrlich ascites tumor cells	20	0
Primary chick fibroblast	3	83
	20	49
	45	42
	70	37
12 day chick erythrocyte	20	100
	45	80
	120	105
	168	90

TABLE XXI

A. The uptake of SRBC coated with different concentrations of antiserum*

Antiserum conc.	Macrophage			1:1 heterokaryon (3 hr)			2:1 heterokaryon (3 hr)		
	A	I	Index	A	I	Index	A	I	Index
0	0.4	0	--	.25	0	--	0	0	--
1/12,500	5.1	3.4	.40	2.7	0.1	.036	N.D.	N.D.	N.D.
1/10,000	1.0	5.4	.84	4.8	1.1	.20	4.6	2.2	.32
1/5,000	0.4	7.0	.95	5.1	4.7	.48	2.7	10.3	.79
1/2,000	0.6	8.2	.92	0.2	9.2	.98	0.3	13	.98

B. The effect of cycloheximide treatment on the ingestion index

Antiserum conc.	No Cycloheximide						Cycloheximide								
	Mac			1:1 Het			Mac			1:1 Het			2:0**		
	A	I	Index	A	I	Index	A	I	Index	A	I	Index	A	I	Index
0															
1/10,000	1	5.4	.85	4.8	1.1	.17	.7	6.7	.90	8.4	1.7	.17	.6	11	.92
1/5,000	.4	7.0	.94	5.1	4.7	.48	.3	5.7	.90	5.9	2.7	.31			
1/2,000	0	7.2	1.00	0	5.9	1.00	0	5.0	1.00	1.2	7.2	.86			

C. The effect of trypsin treatment on the uptake of AbSRBC by 2 day old heterokaryons

Antiserum conc.	No Trypsin						Trypsin					
	Mac			1:1 Het			Mac			1:1 Het		
	A	I	Index	A	I	Index	A	I	Index	A	I	Index
0												
1/10,000	1	6.6	.88	0	0	--	1.2	7.6	.86	3.2	.72	.18
1/5,000	.06	6.0	.99	.3	.2	--	.6	6.6	.91	5.4	1.1	.17
1/2,000	0	6.3	1.00	.4	.6	--	.18	6.4	.97	3.6	4.1	.53
1/1,000	0	6.7	1.00	0	1.6	--	0	5.6	1.00	0	5.8	1.00

*A = mean no. SRBC attached
I = mean no. SRBC ingested
Index = I/A+I

**2:0 = Binucleate macrophage homokaryons

in cycloheximide throughout the three hour period after fusion. Such treatment, however, had no effect on the ingestion index (XXIB). It was also shown that fusion, per se, was not responsible for this distinction between the macrophage and the heterokaryon, since binucleate macrophage homokaryons, 2:0 cells, still reacted like unfused macrophages.

When heterokaryons were examined two days after fusion, few red cells were taken up, even at high antiserum concentration, in agreement with our earlier studies on the disappearance of receptor activity (XXIC). If such heterokaryons were treated with trypsin, to uncover receptor activity, the ingestion index rose with antiserum concentration in the same way as in three hour old heterokaryons.

The results of these and other experiments are summarised in Fig. 25. This shows that two distinct processes which affect the Fc receptor in heterokaryons may be separable by varying antiserum concentration; an early effect occurring within three hours, independent of protein synthesis, and a more delayed effect in which receptor activity is reversibly blocked following protein synthesis.

10. The Distribution of the Fc Receptor. It was now possible to recover full receptor activity in older heterokaryons by trypsinisation, or to preserve such activity in young heterokaryons by cycloheximide treatment. What was the distribution of Fc receptor in heterokaryons under these circumstances?

In order to use AbSRBC for studies of receptor distribution it was necessary to limit the assay to attachment and to increase the number of bound red cells. This could be achieved by incubating cells with AbSRBC for 30 minutes in the cold, followed by four vigorous washes before fixation.

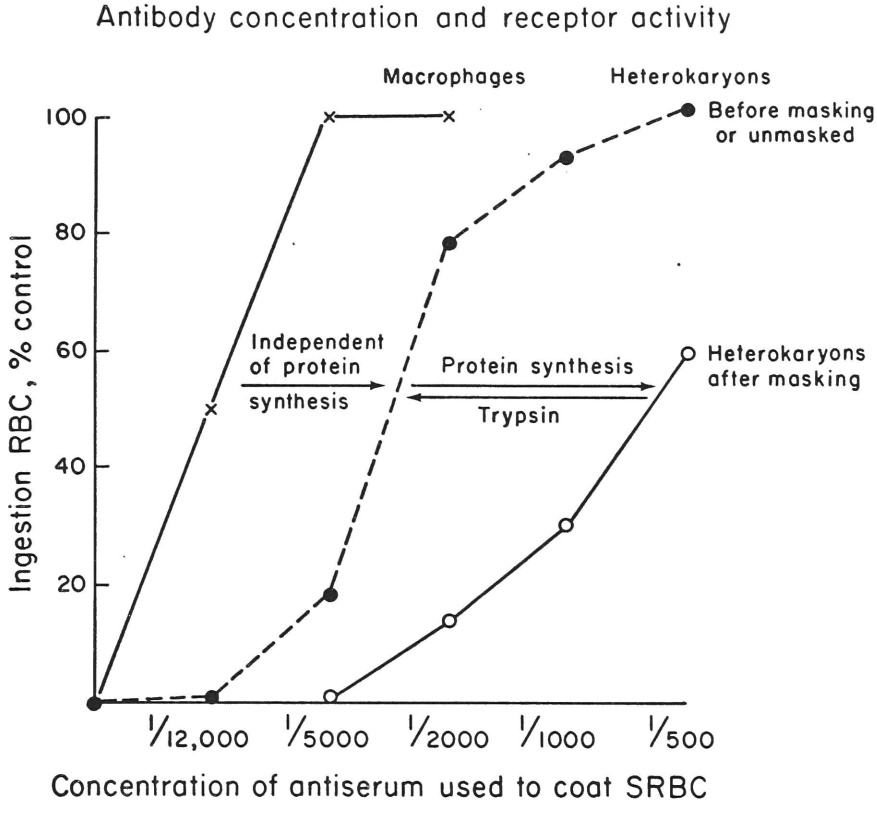

Antibody concentration and receptor activity
Macrophages
Heterokaryons
Before masking or unmasked
Heterokaryons after masking
Independent of protein synthesis
Protein synthesis
Trypsin
Ingestion RBC, % control
100
80
60
40
20
1/12,000
1/5000
1/2000
1/1000
1/500
Concentration of antiserum used to coat SRBC

Figure 25

Such studies were possible in three hour heterokaryons (cycloheximide treated) or in trypsinised heterokaryons, 24 - 48 hours after fusion. In both cases, a diffuse attachment pattern could be observed in those heterokaryons to which many red cells were bound (Fig. 26c, d).

An attempt was made to put this observation on a more quantitative basis. As the number of bound red cells increased, they were found to be distributed over all four quadrants of heterokaryons. It was not possible to show a shift from localised binding to a more diffuse distribution since viral hemadsorption results in the binding of a few AbSRBC to melanoma cells in the early phase of fusion.

Discussion

The Fc receptor offers several advantages as a membrane marker in heterokaryons. It can be assayed in individual cells and dosage effects correlated with cell composition. The receptor is stable under a variety of conditions, which include treatment with trypsin and inhibition of protein and RNA synthesis. Under these conditions sensitised red cells still attach to macrophages and ingestion is unaffected. Heterokaryons performed even better than controls after rather prolonged treatment with inhibitors.

Sendai virus could perhaps itself affect the macrophage receptor. The receptor is, however, insensitive to viral neuraminidase and the homokaryons and unfused cells in the same preparation act as a built-in control for virus action. It is also important to distinguish between receptor activity and viral hemadsorption. Melanoma cells did bind AbSRBC within two hours of virus treatment, especially if this step was carried out in the cold. All phagocytic assays were therefore performed after this time, when melanoma cells no longer bound any erythrocytes. The incubation step in 199 + 10% NBCS, at 37^{o}C, provided another

Figure 26 (a-d)

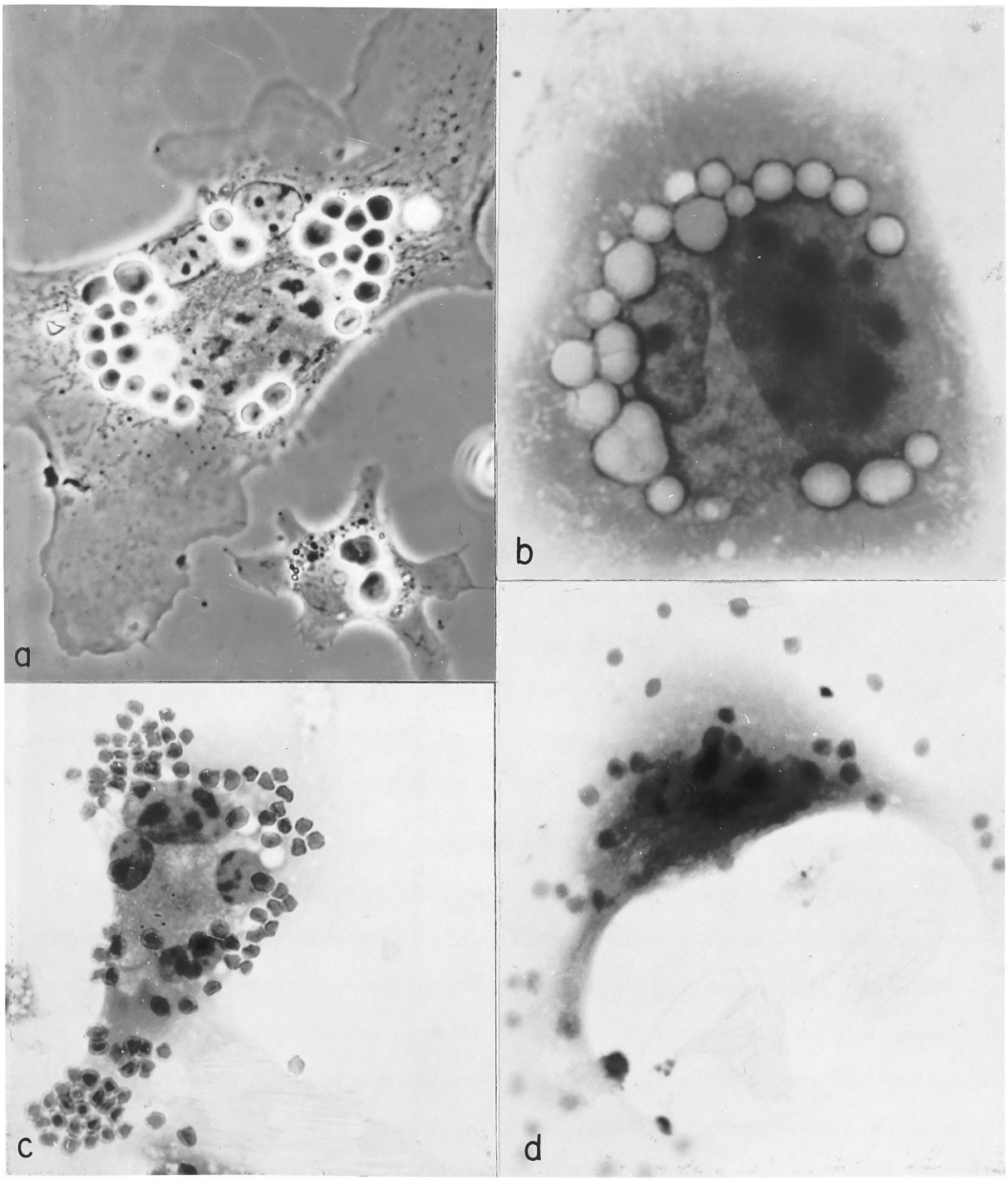
Uptake of AbSRBC by heterokaryons under a variety of conditions.

Fig. 26a. A two day old heterokaryon ingests numerous red cells after treatment with trypsin to unmask the phagocytic receptor. Phase contrast x 800.

Fig. 26b. A nine hour old heterokaryon displays active ingestion of red cells after eight hours treatment with cycloheximide to prevent masking. Heterokaryons are typically well spread after such treatment. Stained preparation. x 1500.

Fig. 26c. AbSRBC attach diffusely to a 3:1 heterokaryon which has been treated with cycloheximide for three hours after fusion. Stained preparation. x 600.

Fig. 26d. AbSRBC attach diffusely to a 1:1 heterokaryon which has been treated with trypsin one day after fusion. Stained preparation. x 800.


a
b
c
d

safeguard since this treatment promotes the elution of hemadsorbed red cells (Watkins and Grace, 1967). Conceivably, hemadsorbed red cells would then be ingested by macrophages, but elute from melanoma cells. We can dismiss this possibility since macrophages ingested a constant number of red cells during the time viral hemagglutinin disappeared from the cell surface.

Macrophages may carry adsorbed gamma globulins on their plasma membrane (Nelson, 1969). Trypsin treatment, which has been reported to enhance phagocytosis by macrophages, could remove such antibody from the cell surface (Kossard and Nelson, 1968). In the present studies unfused macrophages did not take up significantly more AbSRBC after treatment with trypsin. In any event, the evidence suggests that the heterokaryon receptor becomes masked by membrane proteins which can be regenerated by the heterokaryon after treatment with trypsin. The masking process is prevented by inactivating the melanoma nucleus with ultra-violet light or by substituting an inactive chick red cell nucleus for it (Harris et al., 1969). The melanoma nucleus is therefore ultimately responsible for masking the macrophage membrane receptor as well as inducing macrophage DNA synthesis.

The masking reaction could be due to direct steric hindrance of receptors, but more complex conformational changes in membrane structure are of course possible. The insertion of new melanoma proteins into the heterokaryon membrane could, for example, displace the Fc receptor into crypts which are then inaccessible to the sensitised red cells. The masking proteins probably belong to the melanoma cell coat since they are susceptible to proteolytic digestion, without loss of cell viability. Mild digestion with enzymes can unmask several cell surface receptors and antigens in a similar fashion. Proteases have been used to augment or reveal red cell antigens (Springer, 1963)

as well as cell receptors which react with plant lectins like wheat germ agglutinin (Burger, 1969a) or concanavalin A (Inbar and Sachs, 1969). Similarly, SV-40-transformed tumor cells possess surface (S) antigens which become demonstrable in untransformed cells after mild proteolytic treatment (Hayry and Defendi, 1970).

Neuraminidase did not reactivate Fc receptor activity, although it may enhance non-specific phagocytosis (Weiss et al., 1969) and perhaps unmask "immunogenicity" in malignant cells (Currie and Bagshawe, 1969) or trophoblast (Currie, 1968). This failure was not due to the use of inactive enzyme.

We do not know the nature of the masking protein(s) nor how widely it is distributed over the cell surface. Preliminary studies suggest that the macrophage ATPase can also be unmasked by mild proteolysis, so that masking is probably not unique to the Fc receptor. Ehrlich ascites tumor cells masked phagocytic function in heterokaryons even more rapidly than the melanoma cells. Ehrlich ascites tumor cells also suppress the antigens of other cells in hybrids (Klein et al., 1970; Murayama-Okabayashi et al., 1971), perhaps by a similar masking mechanism. Fusion between macrophages and embryonic chick fibroblasts brought about a very slow decline in Fc receptor activity. More cells have to be tested to determine if the masking property is associated with malignancy, rate of cell growth or cell maturity.

Morphologic observations suggested that both ATPase and Fc receptors spread out over the heterokaryon cell surface after initial fusion. This phenomenon could be related to the observation that heterokaryons and macrophages differ in taking up SRBC coated with variable amounts of antiserum. More antibody is needed to promote binding and ingestion by three hour heterokaryons. This difference is not a result of fusion, per se, since macrophage dikaryons resemble

macrophages in this respect. Masking can be excluded at this early stage since it can be prevented by cycloheximide or reversed by trypsin and yet yield heterokaryons with similar properties.

The interpretation of these data is complex, but an early change in receptor function must occur when a macrophage becomes a heterokaryon. One possible explanation is that the density of receptor molecules falls, due to spreading and dilution, as a macrophage fuses with the larger melanoma cell. Since heterokaryons eventually take up the same number of red cells (Fig. 25), it is likely that the heterokaryon initially carries the same number of receptor molecules, but distributed over a larger surface area than before. Such an interpretation makes assumptions about the role of receptor density in binding AbSRBC. No detailed information is available on the distribution, density or binding mechanism of the Fc receptor, but two related phenomena can be cited. Immune complexes of polyvalent haptens bind significantly better to macrophages than those of mono- or divalent haptens (Phillips-Quagliata et al., 1969). This increase could be due to multiple attachment sites rather than to allosteric changes in the antibody. There is good evidence that cytotoxic reactions are also strongly influenced by cell surface antigen density, apparently because IgG doublets have to be present to bind complement (Cohen, 1968). Although such a requirement has not been demonstrated in binding AbSRBC to the Fc receptor, it seems reasonable to postulate that the higher antibody concentration on red cells helps stabilise the interaction between red cell and the heterokaryon receptors.

The studies reported in this chapter therefore suggest that two surface reactions can be distinguished in heterokaryons (Fig. 25), a rapid spreading out of macrophage receptors, independent of protein synthesis, and a slower masking of these receptors as a result of melanoma protein synthesis.

CHAPTER V

THE PREPARATION AND PROPERTIES OF MACROPHAGE-L CELL HYBRIDS

Introduction

The studies reported in Chapter IV showed that the macrophage Fc receptor which had been introduced into a heterokaryon was preserved for some time, but in a masked form. The question remained, could the Fc receptor, a specific property of the macrophage, be synthesised after fusion with a cell which does not express this receptor. No definitive solution to this problem could come from heterokaryon studies since it is not possible to distinguish new from old receptor molecules. We therefore devised a hybrid system to settle this question.

The parent cells, macrophages from the DBA/2 inbred strain of mice and mouse LMTK$^-$ cells, were chosen for the following reasons. Since we were looking for the expression of macrophage properties it was essential to prepare hybrids which would retain the macrophage chromosomes. This could be achieved by fusing mouse macrophages with either mouse or human cells, to obtain conservative or reduced hybrids respectively. In either case they would contain most of the mouse macrophage chromosomes. The only constraints were that we should choose cells which could be eliminated by a selection procedure and which proliferate in vitro. Since macrophages do not divide in culture it was only necessary to set up a half-selection system. As a first step we chose to produce conservative hybrids. LMTK$^-$, a mouse fibroblast cell line which lacks thymidine kinase (TK) activity, met the above requirements and also offered the advantage that no revertants to HAT resistance have yet been reported.

It was also important to prove that we had indeed isolated hybrid cells. Since the LMTK$^-$ cells come from an inbred mouse strain, it was easy to select macrophages from a different inbred strain to provide additional genetic markers. We chose DBA/2 mice since this provided us with suitably different H-2 antigens as well as a simple isozyme marker, phosphoglucose isomerase (PGI). Although mouse chromosomes cannot be distinguished from one another the LMTK$^-$ cells carry chromosome markers and have a low modal number of chromosomes, facilitating studies of hybrid karyotype.

In addition to the Fc receptor and H-2 antigen macrophages have two other membrane markers which can be studied in hybrid cells. LMTK$^-$ cells have very little ATPase activity, like melanoma cells, and the macrophage has a cell specific receptor for complement (Chapter I).

We took precautions to isolate true macrophage hybrids, rather than a chance LMTK$^-$ x DBA peritoneal fibroblast hybrid. Cell cultures from animals with uninflamed peritoneal cavities contain very few fibroblasts. Since macrophages adhere to glass during trypsin treatment, it was also possible to remove contaminant fibroblasts before fusion.

In some studies we compared the hybrid cells with a putative SV 40 macrophage cell line to see if macrophage proliferation, per se, brought about phenotypic changes.

Materials and Methods

1. Cells. Female DBA/2 mice were obtained from the Jackson Laboratory, Bar Harbor. Macrophage cultures were established in the routine way, 1×10^6 cells/22 mm^2 coverslip. After one day's cultivation the monolayers were trypsinized to remove any fibroblasts. The

monolayer was washed 2 x in PD, incubated with TV solution for 10 minutes at 37°C and then washed well with Eagles medium before further cultivation.

LMTK$^-$ cells were provided by Dr. F. Ruddle, Yale University, New Haven. SV 40-macrophages, derived from Balb/c mice, were denated by Dr. Stone, N. I. H., Bethesda.

2. Media. Parental cells were cultured in medium E: Eagles medium; 10 or 20 newborn calf serum, sodium penicillin G (50 u/ml) and streptomycin sulfate (10 µg/ml). Tylocine, 60 µg/ml, or aureomycin, 50 µg/ml (Grand Island Biological Co.), were added on occasion.

Hybrid cells were grown in HAT medium : medium E, supplemented with 1×10^{-4} M hypoxanthine, 1×10^{-5} M aminopterin (K + K Lab.) and 1.6×10^{-5} M thymidine. Glycine (4×10^{-4} M) is present in medium E.

3. Cell Hybridization. Table XXII summarises the production, isolation and study of macrophage-L cell hybrids. Confluent LMTK$^-$ cells were trypsinised and added to two day old macrophage monolayers. After four hours, u-v inactivated Sendai virus was added for one hour. The fused preparations were diluted by trypsinisation 1 day later and placed in HAT. Colonies were detected by 13 days and three clones (LD_3, LD_5 and LD_7) and one mass culture (LD_4) successfully grown up and carried in culture for three months. Only one clone was picked per dish, using the cloning cylinder method of Puck et al. (1956). Cell hybrids were subcultured by trypsinisation at a one-third to one-fifth dilution or stored at -70°C in medium containing 10% glycerol (Wasley and May, 1970).

4. Morphology. Cells were fixed in 1.25% glutaraldehyde and examined by phase contrast microscopy.

5. Chromosome studies were performed 27 - 53 days after fusion,

TABLE XXII

Isolation of Macrophage - LMTK$^-$ hybrids

Days (Fusion = 0)	
-2	DBA/2 macrophages in culture (1×10^6/22 mm^2 c/s)
-1	Monolayer trypsinised
0	LMTK$^-$ cells added (1×10^6/35 mm dish)
	Fused with 1,000 hag^n u. u-v Sendai
1	Trypsinised. Replated in HAT
	HAT changed every 3 - 5 days
13	Colonies detected
18-33	Colonies Isolated → LD3(clone), LD4(mass culture), LD5(clone), LD7(clone)
25-90	Each grown to mass culture and propagated in vitro → ALIQUOT FROZEN; KARYOTYPE; PGI ISOZYMES; SURFACE PROPERTIES ASSAYED (H-2, ATPase, Fc and Complement receptors)

with the help of Dr. C. Ripps of The Rockefeller University. Hybrid LD_3 and LD_5 were examined twice, at 26 and 14 day intervals respectively, the other cells once. Cultures were grown for 1 - 3 days in T-75 Falcon flasks and treated with Velban, 0.025 γ/ml, for three to four hours. The cells were collected by trypsinisation and exposed to 0.075 M KCl for fifteen minutes at 37°C. Preparations were fixed in 1:3 acetic acid : methanol, chromosome spreads prepared, and stained with 2% aceto-orcein (Grand Island Biolog. Co.).

6. Phosphoglucose isomerase (EC 5.3.1.9) phenotypes were determined by starch gel electrophoresis, with minor modifications of the method of DeLorenzo and Ruddle (1969). Fructose-6-phosphate was obtained from Boehringer Mannheim.

Homogenates were prepared from 1 - 1.5 x 10^7 cells obtained by trypsinisation or scraping, in the case of macrophage cultures. The cells were washed in PBS and then disrupted in 0.01 M phosphate buffer, pH 7.0, with the aid of a teflon homogeniser. The homogenates were centrifuged for 60 minutes in a Lourdes centrifuge at 30,000 g and the clear supernatant frozen at -20°C.

7. Surface Properties. a) H-2 antigens were examined by a mixed hemadsorption assay (Espmark and Fagraeus, 1965), schematically shown in Fig. 27. Isoantisera, described in Table XXIV, were donated by Dr. E. Boyse of the Sloan-Kettering Institute, N. Y. Normal mouse serum was obtained by cardiac puncture of NCSR female mice from the Rockefeller colony and used as controls.

A 1% suspension of SRBC in 199 was coated at 37°C for 30 minutes with a 1/640 dilution of heat inactivated mouse anti SRBC antiserum, donated by Dr. R. Franzl of The Rockefeller University. After two washes in 199, the cells were incubated with a 1/20 dilution of a rabbit antimouse IgG serum (Microbiological Associates). These indicator

Mixed hemadsorption assay for H-2 antigen

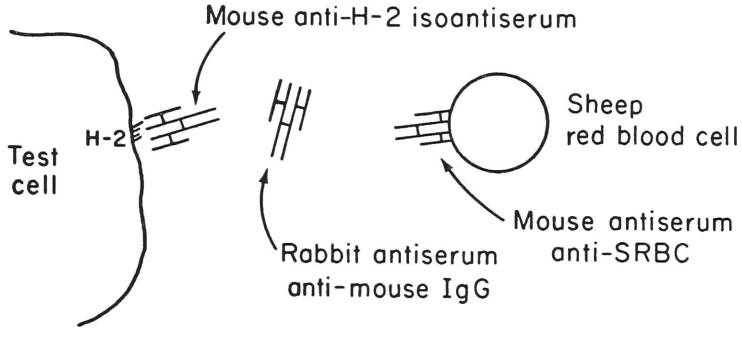


Figure 27

red cells were washed twice in 199 and finally resuspended as a 1/4% cell suspension in 199.

Cells were grown on coverslips for one to four days before assay. Preparations were incubated for one hour at 4°C with serial dilutions of antiserum in 199. The coverslips were then washed 2 x in 199 and incubated with two drops of the indicator red cell suspension for one hour, at 4°C. The cells were washed vigorously, 4 x in 199, and then examined by phase microscopy for rosettes. Preparations were scored 1+ (0 - 25% rosettes), 2+ (26 - 50% rosettes), 3+ (51 - 75% rosettes) or 4+ (76 - 100% rosettes).

b) ATPase assays were performed on non-confluent hybrid or L cells grown for one to four days in 60 mm Falcon plastic dishes while DBA/2 macrophages were grown on 22 mm^2 coverslips. The biochemical method of assay was used (Chapter IV). For the present studies, the incubation mixture contained both mono- and divalent cations. Assays were done in triplicate and the results expressed as $\mu m\ P_i$ released, per μg protein, in thirty minutes.

c) The Fc receptor was assayed by a slight modification of the standard procedure. Sheep red cells were coated with a 1/2,000 dilution of the rabbit antiserum and used immediately. Coverslip preparations were washed 2 x in 199, incubated for 15 - 90 minute periods with antibody coated red cells at room temperature and 37°C, washed well and then examined. Controls were incubated with a similar SRBC suspension which had not been coated with antibody.

d) The macrophage receptor for complement was assayed on coverslips by the procedure of Lay and Nussenzweig (1969a). A 1% SRBC suspension was coated with a 1/10,000 dilution of rabbit amboceptor (Behring Diagnostics) which had been previously heat inactivated at 56° for thirty minutes. The second coat consisted of a 1/10 dilution of fresh

mouse serum, the source of complement. Control indicator red cells were prepared with heat inactivated mouse serum ($56^{o}C$, 30').

Coverslips were washed 2 x in 199, incubated with one drop of a 0.5% suspension of the indicator cells for thirty minutes at $4^{o}C$, washed and examined.

Results

Formation of Hybrid Cells. The macrophage gene for thymidine kinase becomes activated after fusion with $LMTK^{-}$ cells so that complementation results in the exclusive growth of hybrid cells in HAT medium. It was essential to characterise the hybrid cells in more detail before studying their expression of macrophage-specific properties.

1. Morphology and growth. $LMTK^{-}$ cells spread very slowly on glass or plastic (Fig. 29a) and double every 19 hours during the phase of exponential growth. At confluence the cell monolayer has a characteristic cobblestone appearance (Fig. 28a), but further heaping up of cells can occur.

Macrophages spread rapidly on glass or plastic, especially after trypsin treatment. They display prominent membrane ruffling and can move over one another.

Hybrid cells show features of both parents (Figs. 28b, 29b-d). Their nuclei are larger than those of either parent cell. Different hybrid clones resemble one another and all spread extensively within an hour of trypsinisation. The hybrid cells often grow in clusters and frequently overlap one another (Fig. 29d). They double every 36 hours during exponential growth. At confluence the hybrids appear more fibroblastic, without heaping up.

2. Chromosome studies. $LMTK^{-}$ cells have a mean number of 46

Figure 28 (a-c)

Morphology of parent and hybrid cells at or near confluence. Phase contrast.

Fig. 28a. LMTK$^-$ cells show a cobblestone appearance. x 625.

Fig. 28b. Hybrid LD7. The cells show more spreading, but also resemble L cell growth pattern. x 500.

Fig. 28c. Macrophages are well spread, with an irregular cell border. x 625.

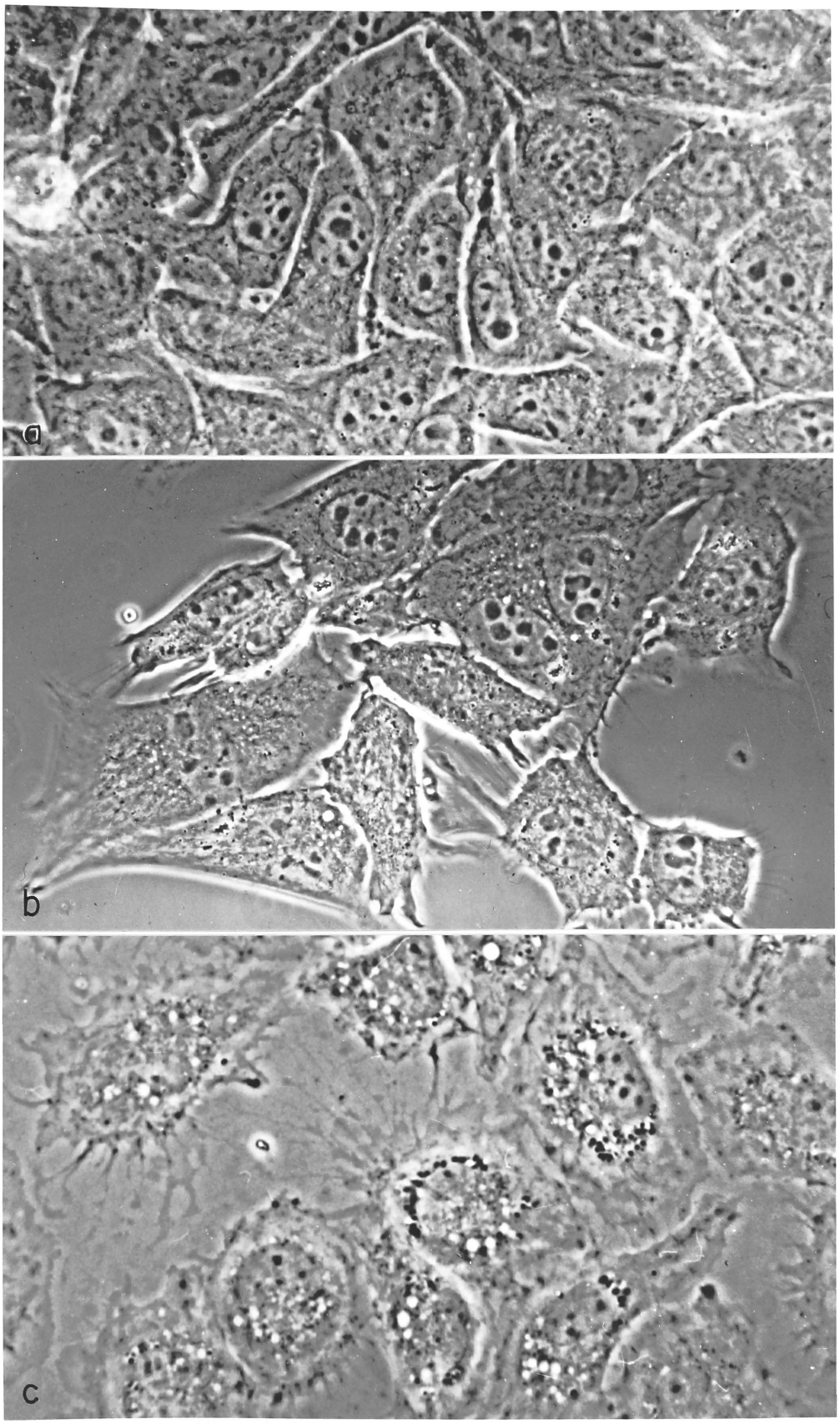

a
b
c

Figure 29 (a-d)

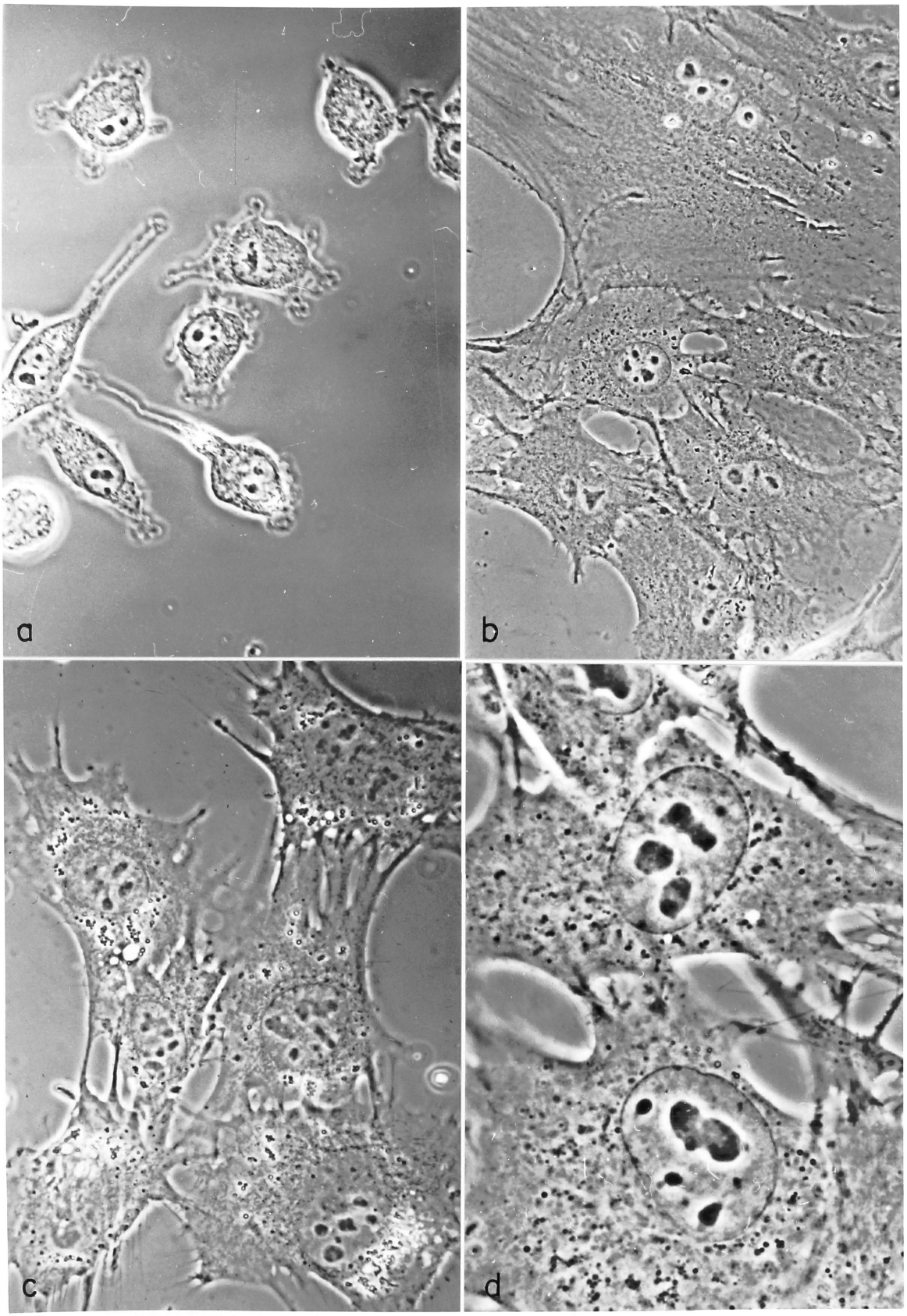
L cells and hybrids cultivated on glass for one day. Phase contrast.

Fig. 29a. LMTK$^-$ cells are poorly spread. x 500.

Fig. 29b. Hybrid LD3 cells are flat and well spread. x 500.

Fig. 29c. Hybrid LD5. Well spread cells with irregular cell border. x 625.

Fig. 29d. Hybrid LD3. Cell processes overlap one another. x 1250.


a
b
c
d

chromosomes, which include 11-13 metacentrics (Table XXIII, Fig. 30). The karyotype of DBA/2 macrophages was not examined, but should consist of the normal diploid mouse complement of 40 acrocentric chromosomes (Green, 1966). The mean chromosome number in the hybrids varied between 73 (LD7) and 88 (LD3) and the range in total chromosome number was much broader for the hybrids than for LMTK$^-$ cells. Only one L cell genome is present in hybrids since 10-13 metacentrics were found in all cases. The hybrids therefore contain 85 - 100% of the chromosomes expected for the fusion of one LMTK$^-$ cell with one macrophage.

3. Isozymes. The Phosphoglucose isomerase phenotypes confirmed the hybrid status of all the LD cells. The LMTK$^-$ cells and DBA macrophages displayed single fast and slow bands, respectively, and all the hybrids showed fast, slow and intermediate "hybrid" bands.

4. Surface properties. (a) H-2. The results of the mixed hemadsorption assay are given in Table XXIV. L cells, which originate from a C_3H mouse, show only H-2k activity (titre 1/3,000). The macrophages, from DBA/2 animals, should have H-2d antigens. They were not examined in the present studies since the indicator cells used here would react with the Fc receptor, irrespective of H-2 genotype. If the Fc fragment on the indicator red cells is removed by treating the IgG with pepsin, H-2 activity of the appropriate genotype can be demonstrated in macrophages (Previous unpublished studies).

All the hybrids showed both H-2k and H-2d activity. The titre varied somewhat, e.g. LD3 cells had a higher H-2k titre than the other hybrid cells (1/10,000 vs 1/3,000). The H-2d titre was 1/3,000 - 1/1,000. The difference between d and k correspond with the different titre which these two antisera display in a cytotoxic assay with lymph node cells. At higher antiserum concentrations all reactions

TABLE XXIII

Chromosomes of macrophage-L cell hybrids

Cell type	Range*	Mean	Metacentrics
LMTK$^-$	42-50	46	11-13
LD3	79-96	88	10-13
LD4	71-91	81	10-13
LD5	75-95	86	10-13
LD7	63-80	73	10-13

*25 metaphases counted per cell line.

Figure 30. Karyograms. (Courtesy of Dr. C. Ripps)

a.) LMTK$^-$. 44 chromosomes, including 13 metacentrics. Note chromosome with double centromere (arrow), possibly due to fusion of two acrocentrics. x 1350.

b.) Hybrid LD7. 72 chromosomes, including 10 metacentrics. x 1250.

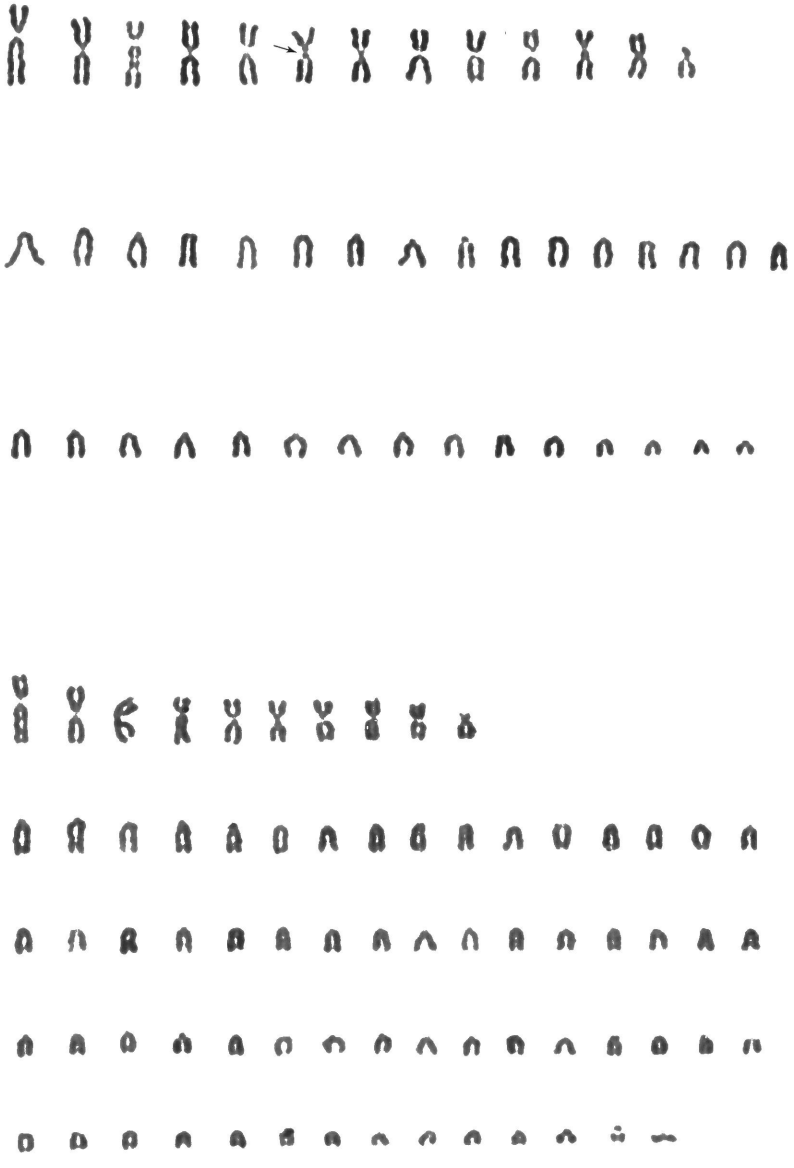


TABLE XXIV

H-2 antigens of macrophage-L cell hybrids*

Cell type	Antiserum	Dilution of antiserum used						
		0	1/10,000	1/3,000	1/1,000	1/300	1/100	1/40
L	**anti-k	--	1+	4+	4+	4+	2+	--
	anti-d	--	--	--	--	--	--	--
LD3	anti-k	--	4+	4+	4+	4+	2+	N. E.
	anti-d	--	--	4+	3+	--	--	--
LD4	anti-k	--	2+	3+	4+	3+	N. E.	N. E.
	anti-d	--	1+	3+	4+	3+	N. E.	N. E.
LD5	anti-k	--	2+	3+	4+	3+	2+	N. E.
	anti-d	--	--	3+	3+	2+	--	N. E.
LD7	anti-k	--	--	2+	3+	2+	1+	N. E.
	anti-d	--	--	2+	3+	3+	1+	N. E.

*Mixed hemadsorption assay. No reaction with normal mouse serum (1/10 or 1/1,000).

**Anti-k = ($C_{57}BL/6$ x Balb) F1, anti BP8(C_3H) = bd anti-k. Pool EE.
Lymph node titre 1/5,000.

Anti-d = C_3H/An anti meth A(Balb/c) = k anti-d. Pool EI.
Lymph node titre 1/2,000.

N. E. = Not Examined.

were diminished, or even abolished, in hybrid as well as L cells. Specificity controls showed that the L cells lacked the d antigen and that normal mouse serum gave a negative result.

(b) ATPase. The ATPase activity of parental and hybrid cells is listed in Table XXV. The hybrids showed some variation in activity in two separate experiments, although agreement for triplicate assays within each experiment was excellent. LMTK$^-$ cells have only 0 - 2% of macrophage ATPase activity, but all the hybrids have intermediate activity, 10 - 23 % . Preliminary experiments suggested that this activity was not Ouabain sensitive, corresponding with the ATPase behavior of macrophages, but more experiments are necessary to confirm this finding.

The SV 40 macrophage ATPase activity was similar to that of the hybrids, viz 22 % of the macrophage control.

(c) Fc receptor. Repeated assays failed to demonstrate Fc receptor activity on any of the hybrids. Cells were assayed during exponential growth or after several days at confluence, to exclude possible variation during the cell cycle. Red cells could bind to L cells or hybrids incubated with SRBC for prolonged periods (two hours) in the absence of antibody, but this binding was suppressed by using AbSRBC.

Experiments were done to exclude a masking phenomenon resembling that found in macrophage-melanoma cell heterokaryons. Hybrids were treated with different doses of trypsin, in the range 0.1 - 250 μg/ml, to ensure that the hypothetical hybrid Fc receptor was not now trypsin sensitive and therefore destroyed during "unmasking". Results were completely negative. Similar studies with a line of "SV 40 macrophages" were also negative.

TABLE XXV

ATPase activity of macrophage-L cell hybrids

Cell Type	mean ± S. D. $\mu M P_i/\mu g$ prot/30' $\times 10^{-3}$	% macrophage activity
DBA/2 macrophages	491±36	100
LMTK$^-$	0±0; 9.6±2.0*	0, 2
LD3	51±3.5; 114±15	10, 23
LD4	77±1.5; 115±10	16, 23
LD5	62±2.6	13
LD7	60±6.0; 64±5.7	12, 13
SV 40 macrophages	110±12	22

*Two separate experiments

(d) Complement receptor. Control macrophages showed 4+ rosette formation when active complement was used. L cells were negative, as were the controls with heat inactivated complement. Macrophages maintained their C binding activity during four days cultivation in vitro. Trypsin treatment (10 - 100 μg/ml, 37°, 30') abolished rosette formation and such cells regained their reactivity one to two days after cultivation in 199 + 20% newborn calf serum.

The hybrids, however, showed no activity before or after trypsin treatment. Studies similar to those searching for the Fc receptor were again completely negative.

The properties of parental cells and hybrids are summarised in Table XXVI.

Discussion

The hybrid nature of the cells obtained in the present studies is confirmed by their karyotype, PGI isozymes and H-2 antigens. It is more difficult to prove conclusively that these are macrophage hybrids rather than peritoneal fibroblast x LMTK$^-$ hybrids, since none of the above markers are macrophage-specific and since, with the possible exception of the ATPase activity, the truly macrophage-specific surface receptors were not demonstrable in the hybrid cells. Precautions were taken to prevent fibroblast contamination of macrophage cultures. There is no preferential fusion of fibroblasts with L cells. Macrophage-melanoma cell heterokaryons can give rise to slow growing hybrid cells (Chapter II), so there is no reason to expect hypothetical fibroblast x L cell heterokaryons to grow preferentially. Finally, since several independent clones were isolated, we feel confident that the hybrids are indeed macrophage hybrids.

The hybrids conserved 85 - 100% of the sum of the chromosome

TABLE XXVI

The properties of parental cells and hybrids

TRAIT	Macrophage	LMTK$^-$	Hybrids
In vitro proliferation	-	+	+
Thymidine kinase	+	-	+
Karyotype (mean)	40	46	73-88
Phosphoglucose isomerase isozymes	slow	fast	slow, fast and intermediate
Surface properties			
Spreading	+	-	+
H-2	d	k	d,k
ATPase (percent)	100	0-2	10-23
Fc receptor	+	-	-
Complement receptor	+	-	-

complement of two parent cells, like other mouse x mouse hybrids (Ruddle, 1970). It is not possible to decide which cell's chromosomes were lost in the macrophage-L cell hybrids. In some hybrids chromosomes seem to be lost preferentially from the genome of the parent with the longer generation time (Migeon and Childs, 1970). This would suggest that mostly macrophage chromosomes were lost from the macrophage-L cell hybrids. However, since confluent L cells, which should be mainly in G_1, were chosen for fusion, it is likely that the macrophage and L cell nuclei of 1:1 heterokaryons entered S in synchrony (Chapter III) and that macrophage chromosomes need not have been lost preferentially during the early divisions. We find evidence for the expression of macrophage TK, PGI, H-2 and probably ATPase genes. It is therefore unlikely that the loss of the two macrophage specific surface receptors is due to the loss of their genes, since this would have had to occur selectively, and in several independent hybrids.

The surface properties of these hybrids are of great interest. Many types of cell show plasma membrane ATPase activity, but the macrophage is a particularly rich source and the enzyme requires only divalent cations for full activity. The ATPase of the hybrids, expressed as specific activity, lies intermediate between that of the macrophage and L cell parents. Similar intermediate levels of activity have been observed in several different hybrid systems (Table I).

The absence of the macrophage Fc and complement receptors poses an interesting problem. We assume that the chromosomes which carry the genes for these receptors have not been lost. The receptors are not masked, nor can they be brought to light by simply manipulating the growth phase or density of the cell population. Since the macrophage H-2 antigen <u>is</u> expressed in the hybrids, the absence of the other membrane receptors is selective. The failure to detect only certain

membrane markers has not been demonstrated in other hybrid cells. H-2 and species antigens seem to be expressed whenever their genes are present, except for Ehrlich ascites cell hybrids which suppress surface markers non-selectively, probably as a result of local membrane effects (Klein et al., 1970). Some of these Ehrlich hybrid cells retain their H-2 genes, since the antigen occasionally reappears, perhaps associated with chromosome loss.

We have no evidence to account for the selective "absence" of macrophage membrane markers in our hybrids. It is rash to equate non-detectability with true absence, judging from previous experience. One possible explanation is that dividing macrophages do not express these receptors. Non-dividing macrophages maintain these receptors in culture over several weeks and it is likely, but not proven, that receptor synthesis takes place. The only dividing macrophage lines currently available are derived from SV 40 transformed primary cultures. In our hands such a line did not demonstrate receptor activity, although claims have been made for non-specific phagocytic activity in these cells (Stone and Takemoto, 1970). It can, however, be argued that the transformation process disturbs membrane architecture so that we still don't know if proliferative growth, per se, causes loss of the receptors. ATPase activity in the SV 40 macrophages resembled that of the hybrids, perhaps a point in favor of their macrophagic nature.

Cell-specific properties are often lost during the course of long term cultivation in vitro (Krooth and Sell, 1970). Hybrid cells also commonly lack cell-specific traits expressed by their parent cells in culture (Table I). The selective absence of such traits in hybrid cells may prove to be important in understanding how cells come to express cell-specific properties.

CHAPTER VI

GENERAL DISCUSSION

Nuclear activation in heterokaryons closely resembles the dramatic changes which occur when frog somatic cell nuclei are transplanted into egg cytoplasm (Gurdon and Woodland, 1968). These include rapid swelling, chromatin dispersion and induction of nucleic acid synthesis. The injected nucleus plays a passive role under the influence of its new cytoplasmic environment. The cytoplasmic control of nuclear function has also been investigated in subcellular systems (Friedman and Mueller, 1969; Mueller, 1970). Cytoplasmic factors, including proteins, are necessary to support DNA synthesis in isolated nuclei. The results obtained with "nucleus" and "cytoplasm" should not be equated with events which occur in vivo, however, since loosely bound proteins may be extracted from the nucleus when cells are disrupted in an aqueous environment (Allfrey, 1970). Thompson and McCarthy (1968) have claimed that a heat stable cytoplasmic factor from HeLa or regenerating liver cells could stimulate DNA synthesis in isolated erythrocyte or liver cell nuclei, but their system is rather inefficient and not well characterised.

Studies of nuclear activation in heterokaryons suggest that some cytoplasmic proteins are transported into these nuclei and account, in part, for their increase in dry mass. Newly synthesised histones probably migrate into reactivated nuclei during DNA replication (Robbins and Borun, 1967; Mueller, 1970). The exchange of proteins between cytoplasm and nucleus is a widespread, poorly understood phenomenon (Zetterberg, 1970; Goldstein and Prescott, 1967). Labeled proteins which have been previously synthesized by, or injected into, frog egg cytoplasm can be selectively concentrated in brain nuclei

introduced into these eggs along with inhibitors of protein synthesis (Arms, 1968; Merriam, 1969). Evidence that some nuclear proteins derive from the cytoplasm does not exclude the possibility that others may be made in the cell nucleus itself (Allfrey, 1970; Goldstein, 1970). In any event, these cytoplasmic proteins must be extremely heterogeneous and also play a role in processes unrelated to DNA replication. Future progress in characterising some of these proteins may be achieved by exploiting their affinity for DNA (Alberts, 1970).

The nuclear envelope provides a permeability barrier controlling the traffic of ions, as well as proteins, between cytoplasm and nucelus (Fry, 1970). The swelling of dormant nuclei in heterokaryons or after nuclear transplantation must surely be accompanied by a change in permeability. Swelling is necessary to induce both RNA and DNA synthesis in such nuclei, perhaps because ionic changes in the nucleus alter chromatin structure. The physical state of chromatin is determined by interactions between DNA, histones and nuclear phosphoproteins (Allfrey, 1970). A rapid change in histone acetylation accompanies gene activation, e. g. after phytohemagglutinin stimulation of lymphocytes and probably also occurs during activation of nuclei in heterokaryons.

A major unsolved problem in cell biology has been to discover how nuclei change function during cell differentiation. The classic transplantation experiments of Gurdon and others (1968) showed that the nucleus of a differentiated cell retains all the genes of an organism. What then determines differential gene activity in a cell? It was hoped that cell fusion could contribute a powerful tool to study this problem. Relatively non-specific, large scale changes in gene activity do occur in heterokaryons, but no differentiated cell function, e. g. hemoglobin synthesis, has yet been reactivated. This could be the result of subtle

incompatibility between species specific biochemical processes in fused cells or relate to problems of in vitro culture. It is also true that no one has adequately studied cells like myoblasts, which can differentiate in vitro, in a fusion system. Finally, only the egg may be able to provide the unique environment which can bring about true redifferentiation of a somatic cell nucleus.

The Spreading Out of Macrophage Membrane Receptors

Cell fusion makes it possible to join two different types of cell membrane at a given time and observe the subsequent reorganisation of membrane structure. The evidence for spreading out of macrophage receptors over the heterokaryon surface is incomplete. The kinetics or extent of this process could not be gauged. A suitable melanoma cell membrane marker, e. g. H-2, should be studied at the same time as the macrophage receptors, but double detection systems are complicated by the reactivity of the macrophage with γ globulin. The present studies were performed at only a low level of resolution.

Frye and Edidin (1970) have provided further evidence for an intramembrane intermixing process in newly formed heterokaryons. They used a double labelling, fluorescent antibody technique to detect cells which became mosaic for mouse H-2 and human species antigens. Total intermixing of fluorochromes had occurred 40 minutes after adding Sendai virus and was also observed in some human-mouse hybrids. They used a variety of inhibitors to try and distinguish between several mechanisms: a very rapid metabolic turnover of antigen; the integration of new units from a pool of cytoplasmic precursors into the plasma membrane; diffusion of antigens in the plane of the membrane or movement via the cytoplasm to different membrane sites. Inhibitors of protein synthesis did not affect the formation of mosaic

cells, even after pretreatment for six hours, nor did the use of dinitrophenol and sodium fluoride which, in combination, depleted cell ATP content by 80 per cent. At lower temperature mosaic cell formation was prevented and a plot of per cent mosaic cell vs temperature produced a sigmoidal curve. The chemistry, synthesis, turnover or precursor pools of these antigens are unknown, but observations that inhibitors of protein synthesis can prevent the synthesis of membrane proteins (Warren and Glick, 1968) argue against the existence of an independent, drug insensitive compartment in heterokaryons. It is unlikely that cells become mosaic as a result of new antigen synthesis, although rapid sugar addition reactions are not completely excluded. The authors interpreted their findings as evidence for a temperature dependent diffusion process within the membrane.

It is interesting to consider the implications of such a process for concepts of membrane structure. Many attempts have been made in recent years to isolate the plasma membrane, solubilise its protein and study its organisation with the aid of refined physical methods (Winzler, 1970; Chapman, 1970). Membrane proteins and lipids are grossly heterogeneous and there is no good evidence that different membranes share important "structural" type proteins. The permeability barrier presented by a membrane is due to a quasi-continuous bilayer of lipid. A substantial fraction of phospholipids and proteins can change structure independently of each other (Glaser et al., 1970), but a class of lipid-protein interactions is probably important for the function of many membrane enzymes (Singer, in press). Arguing from observations of this kind, as well as thermodynamic principles, Singer and his coworkers proposed a lipid-globular protein mosaic model for membrane structure, with hydrophobic lipids and nonpolar amino acids in the hydrophobic interior and ionic lipids, charged residues of proteins and sugars in contact with the aqueous environment.

Several studies suggest that membranes and phospholipid bilayers could contain the type of fluid structure which makes credible the hypothesis that membrane antigens diffuse within a heterokaryon plasma membrane. Phospholipid-water mixtures can form a variety of stable structures, in vitro, which undergo phase transitions when the temperature is shifted (Luzzatti, 1968). The hydrocarbon chains of phospholipids are probably liquid at 37°C and cells may control the necessary degree of membrane fluidity over a wide range of conditions, e. g. by utilisation of saturated vs unsaturated fatty acids or by their cholesterol and sphingomyelin content (Chapman, 1970). Fluid regions can be detected in mycoplasma membranes by differential calorimetry (Steim et al. , 1969) and in myelin by the mobility of electron spin labels within the membrane (Hubbell and McConnell, 1969). Low angle X-ray diffraction studies of photopigment molecules in frog retinal receptor disk membranes suggest that the pigment molecules float in a liquid-like environment (Blasie and Worthington, 1969).

Studies on membrane formation are also compatible with such a concept (Siekevitz, 1970). Lipids and proteins, including enzymes, seem to be synthesised independently, in multistep fashion, during phases of rapid membrane growth. New enzymes and lipids (Mindich, 1970) are introduced throughout the preexisting membrane framework.

Masking and Cell Membranes

It is interesting to compare the present findings with those made in chick erythrocyte-mouse fibroblast heterokaryons (Harris, 1970). The kinetics of disappearance of chick species antigens from these heterokaryons resembles that of the Fc receptor and u-v irradiation of the chick red cell before fusion also failed to prevent its disappearance. This process also depended on cell metabolism since incubation

at lower temperature delayed the loss of antigens. It is unlikely, however, that this loss was due to masking, since these antigens reappear spontaneously and accumulate progressively. Do these two heterokaryon systems differ because of the particular receptor under study or because of the cells involved? Both possibilities can be tested.

If we knew the chemistry of the Fc receptor it would help to explain the nature of the masking process. A unified picture has recently emerged from biochemical, genetic and immunologic studies of related blood group antigens (Watkins, 1966). The individual specificity of many membrane receptors resides in glycoproteins and glycolipids which contain oligosaccharide units of unique sequence and conformation. Sugar residues are added sequentially to appropriate precursors by specific transferases which are genetically determined. Many cells have a carbohydrate-rich cell coat which can be removed by enzyme treatment, but its chemical nature is poorly defined (Martinez-Palomo, 1970).

Does material have to be removed from the heterokaryon surface to achieve unmasking, or does cleavage of particular peptide bonds bring about a reorganisation in membrane structure similar to that postulated for wheat germ agglutinin receptors (Burger, 1969b). Studies with these receptors suggest that such differences in membrane structure play an important part in regulating cell growth. The receptors are briefly exposed during mitosis in normal cells and then become cryptic (Fox et al., 1971). Malignant cells display the same receptors permanently. Normal growth can be restored by covering the agglutinin sites on the tumor cell (Burger and Noonan, 1970) whereas mild proteolytic treatment can induce DNA synthesis in resting normal cells (Burger, 1970).

The masking reaction also has potential significance in tumor-

host relationships (Klein, 1969). The expression of tumor specific antigens determines whether a tumor is immunogenic or acts as target to the host's immune system. Patients with tumors often have circulating antibodies which enhance tumor growth by masking tumor antigens (Hellstrom and Hellstrom, 1969). The macrophage heterokaryons illustrate another mechanism by which a tumor cell could alter its membrane properties in such a way that it would escape immunological surveillance (Wilson, 1970). Landschutz ascites tumor cells, for example, become more immunogenic after neuraminidase treatment and can protect mice against an otherwise lethal challenge with untreated cells (Currie and Bagshawe, 1969). Tumor cells which can diminish their antigenicity would be expected to have considerable selective advantage in vivo and a masking phenomenon could also be invoked to explain tumor progression.

The Expression of Membrane Receptors in Hybrids

As a rule cells express species and histocompatibility antigens during long term culture in vitro. The present studies confirm that H-2 antigens provide useful and sensitive markers to identify cell hybrids. Weiss and Green (1967), in an attempt to localise the chromosomes which specify human species antigens, showed that these antigens could be detected in reduced human-mouse hybrids containing a very small number of human chromosomes (< 4) and that genes for such antigens appear to be widely distributed among different human chromosomes. The "absence" of one parent's species antigens in a hybrid, however, does not rule out the presence of other genes from this parent (Schwartz et al., 1971).

Macrophage-L cell hybrids do not express their macrophage-specific receptors. In contrast, neuroblastoma x L cell hybrids pre-

serve the ability to perform a specialised membrane function viz. electrical excitability (Minna et al., 1971). Although the membrane components which produce an action potential have not been identified, several cation transport systems probably have to be present in a complex structure.

The failure to express macrophage-specific receptors in hybrids could be due to incomplete receptor synthesis if the appropriate transferase were absent. Hakomori (1968a, b) found that the glycolipids of transformed cells lacked their terminal sugars. It has also been reported that human adenocarcinoma cells may lose A and B antigens in culture, while expressing H, a precursor molecule (Winzler, 1970).

In the final analysis, cell surface individuality depends on the arrangement of receptors as well as their chemical nature. Boyse and Old (1968), and their collaborators, have explored the topography of cell surfaces with the aid of serologic and immuno-electron microscopic methods. Many membrane antigens are confined to discrete regions and occupy a characteristic proportion of the total cell surface. They also postulate the widespread existence of differentiation antigens, similar to those shown on thymocytes and skin cells, which, together with differential surface structure, could mediate cell recognition phenomena.

Cell hybrids provide a suitable tool to test concepts like these. Their mosaic membrane properties should be amenable to surface mapping and genetic analysis. Macrophage hybrids are particularly useful since they carry the genes for differentiation as well as non-specific membrane receptors. If, for instance, macrophage receptors are undetectable in conservative mouse-mouse hybrids because non-macrophage chromosomes are also present, will they reappear in reduced mouse macrophage x human non-macrophage hybrids as

the human chromosomes are lost? This, and many other questions raised in the course of the present experiments, will be pursued in the future.

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