MACROPHAGE–MELANOCYTE HETEROKARYONS*

I. Preparation and Properties‡

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The fusion of animal cells to form heterokaryons and hybrids makes it possible to gain new insights into the control of nuclear and cytoplasmic functions (1–4). Through the use of Sendai virus one may fuse cell partners which differ widely in their capacity to synthesize DNA as well as in other more differentiated activities (5, 6).

For the present experiments we have chosen the mouse peritoneal macrophage and a strain of mouse melanocyte. The macrophage does not synthesize DNA under a variety of cultural conditions and appears to be blocked in the G_0 period (7–9).

The macrophage has other properties which make it a useful partner in cell fusion experiments. These include the cultivation of pure populations in vitro, the ability to ingest antibody-coated red cells, and the presence of a highly active adenosine triphosphatase (ATPase)[1] which is dependent upon divalent cations (10–13). In addition the macrophage is able to synthesize significant amounts of lysosomal enzymes, e.g. acid phosphatase, providing another useful cytochemical marker (10).

A mouse melanocyte cell line was chosen for fusion with macrophages since these cells proliferate vigorously in vitro, lack the specific macrophage markers, and fuse readily with mouse peritoneal macrophages.

When macrophages are fused with melanocytes, the macrophage nuclei undergo striking changes, including the initiation of DNA synthesis. All

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* In this paper, the abbreviation 1:1 heterokaryon refers to a cell with one macrophage nucleus and one melanocyte nucleus; 2:1 heterokaryon to a cell with two macrophage nuclei and one melanocyte nucleus, etc.

[1] *Abbreviations used in this paper*: ADP adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; BSA, bovine serum albumin; CTP, cytidine triphosphate; DM, melanocyte culture medium, consisting of Dulbecco's medium with 10% calf serum and 10% tryptose phosphate broth; GTP, guanosine triphosphate; ITP, inosine triphosphate; 199 M, see first paragraph of Materials and Methods; P_i, inorganic phosphate ion; PBS, phosphate-buffered saline; PD, PBS without Ca^{++} and Mg^{++}; TCA, trichloroacetic acid; TV, 0.25% trypsin and 0.05% Versene in PD.

macrophage specific markers could be detected in heterokaryons after fusion, but disappeared from these cells in an irreversible fashion. The present paper describes the preparation and properties of macrophage–melanocyte heterokaryons with special reference to the fate of macrophage markers.

Materials and Methods

Media.—Macrophage culture medium consisted of medium 199 (Microbiological Associates, Inc., Bethesda, Md.) and 10 or 20% newborn calf serum (Grand Island Biological Co., Grand Island, N.Y.) which had been heated for 30 min at 56°C and passed through a Millipore filter, pore size 0.45 μ. Penicillin G (Eli Lilly & Co., Indianapolis, Ind.) was added at 200 units per ml. The complete medium will be referred to as 199 M.

Melanocyte 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 (14) and PD to PBS without Ca^{++} and Mg^{++}. TV consisted of 0.25% trypsin (Nutritional Biochemicals Corp., Cleveland, Ohio) and 0.05% Versene in PD. Glutaraldehyde (Fisher Scientific Co., Fair Lawn, N.J.) was used as a fixative at a concentration of 1.25% in PD.

Reagents and Isotopes.—The materials used in these experiments were purchased from the following sources: uridine-6-^{3}H, 10.4 Ci/mM and thymidine methyl-^{3}H, 2 Ci/mM, New England Nuclear Corp., Boston, Mass.; L4 emulsion, Ilford Ltd., Essex, England; deoxyribonuclease I, Worthington Biochemical Corp., Freehold, N.J.; dextran sulfate, mol wt 2×10^6, Pharmacia Fine Chemicals, New Market, N.J.; adenosine triphosphate (ATP), sodium salt, Mann Research Labs. Inc., New York, tris(hydroxymethyl)aminomethane salt, Sigma Chemical Co., St. Louis, Mo.; sodium β-glycerophosphate, Eastman Kodak Co., Rochester, N.Y.; ouabain, Sigma Chemical Co.; sodium β-naphthyl acid phosphate, Dajac Laboratories, Philadelphia, Pa.; cyclic adenosine monophosphate (AMP), Calbiochem, Los Angeles, Calif.; AMP 2′3′, Mann Research Labs; inosine triphosphate (ITP), adenosine diphosphate (ADP), guanosine triphosphate (GTP), cytidine triphosphate (CTP), AMP 5′ and glucose-6-phosphate were all obtained from the Nutritional Biochemicals Corp.

Cells.—Cells were obtained as follows: melanocytes, nonpigmented clone NPN87, isolated by Dr. S. Silagi of The New York Hospital from line B16 cells (15); baby hamster kidney cells (BHK21-F) from Dr. K. V. Holmes, The Rockefeller University; HeLa, L2 and L929 cells from Dr. L. Stuurman, The Rockefeller University.

Fresh sheep red blood cells (Animal Blood Center, Syracuse, N.Y.) were washed 3 × in PBS and suspended at 1×10^9 cells per ml. Rabbit anti-sheep red cell antiserum was a gift from Dr. M. Rabinovitch, The Rockefeller University. The antiserum contained mainly 7S antibody, at a titer of 1:5760.

Method of Cell Culture.—

Macrophage cultures were set up according to the method of Cohn and Benson (10), with some modifications. Female mice from the NCS colony maintained at The Rockefeller University and weighing 25–30 g 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, Los Angeles, Calif.). Each mouse yielded about 2×10^6 cells from which seven cover slips could be prepared. After 1 hr 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.

Melanocyte culture.—Cultures were grown in DM, harvested with TV and 1% of the cells replated weekly. Cells for fusion experiments were routinely cultivated overnight to provide a standard, exponentially growing population of cells.

Microscopy.—Coverslip preparations were fixed in glutaraldehyde for 10 min at 4°C and examined by oil immersion phase-contrast microscopy using a Zeiss Ultraphot, Model II. Photographs were taken using 4 × 5 in. 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 hens' eggs, partially purified and stored, following the method of Harris and coworkers (5).

Hemagglutination titers were determined using serial dilutions of virus in PBS containing 0.5% bovine serum albumin (BSA) (16). A 0.5% suspension of chick red blood cells was incubated with virus for 70 min at 4°C.

Viral infectivity was assayed in 10-day fertile hens' eggs. 10-fold dilutions of virus were injected into eggs and the hemagglutination titer of the allantoic fluid measured after incubation for 2 days at 35°C. The ED_{50} was calculated using the Reed-Muench formula (17).

Ultraviolet irradiation was used to inactivate virus. Concentrated virus was thawed immediately before use and irradiated with a Sylvania germicidal lamp (G15T8) for 9 min 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 (5).

Treatment with unirradiated Sendai virus leads to the production of viral hemagglutinin in macrophages as well as in melanocytes, although this process takes longer in the macrophage. The treatment with ultraviolet irradiation destroyed 8 log units of the viral infectivity, as measured in hens' eggs, without significant reduction of fusion activity. Irradiation of virus effectively abolished its ability to produce viral hemadsorption in macrophages as well as in melanocytes.

Cell Fusion Technique.—

Macrophage homokaryons: Macrophages were cultivated for 1 day or longer at a cell density of 2×10^5 cells per cm^2. Cover slips were washed twice with 199 M, 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 cover slip. After 1 hr incubation at 37°C, the cover slips were washed twice with 199 M and further incubated in 199 M.

In the procedure described above macrophages were cultivated on a cover slip 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.

Macrophage-melanocyte heterokaryons: The melanocyte was chosen as the fusion partner for macrophages after preliminary experiments with several lines, including HeLa, L2, L929, and BHK 21-F. Melanocytes 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 melanocytes was obtained by washing a monolayer of cells twice with PD, followed by TV treatment for 3 min 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. 10–20,000 melanocytes were added to the macrophage monolayer on each cover slip and allowed to settle. After 1 hr of incubation, the cover slips were washed and either treated immediately with virus or cultivated in 199 M for some hours before fusion.

Isolation of heterokaryons by means of trypsinization: For certain studies, large numbers of 1:1 heterokaryons were required which were free of melanocytes. 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 melanocytes differently. Whereas melanocytes 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 melanocytes.

Macrophages (about 5×10^6 cells) were cultivated in 15 cm^2 T flasks for 1–3 days. One million melanocytes were resuspended in 2 ml of 199 M and allowed to become attached to the macrophage monolayer for 1 hr at 37°C. After rinsing the cells twice with warm 199, 2 ml of 199 M, containing 5000 h.a.u. of inactivated virus, were added to the monolayer and the culture kept at 37°C for 1 hr. 2–4 hr after the addition of fresh medium, the preparation was washed twice with PD and incubated for 5–10 min 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 cover slips at the desired cell density. After the heterokaryons had spread out, the nonattached cells, mainly melanocytes, 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.

Dextran sulfate-laden macrophages: On occasion dextran sulfate was used as a macrophage marker. 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 1 day and then exposed to 10 μg/ml dextran sulfate in 199 M for 1–2 days. Free dextran sulfate was removed prior to the addition of melanocytes. After the melanocytes 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 melanocytes, 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.

Properties of Heterokaryons.—Cover slip preparations of heterokaryons were examined at various times after fusion by the following procedures:

(*a*) *RNA synthesis* was studied by radioautography using tritiated uridine as tracer. Duplicate preparations were incubated for 1 hr in 199 M containing 10 μCi/ml uridine. Coverslips were washed twice in 199, fixed in methanol, and processed for radioautography. Grains were counted for quantitation of RNA synthesis.

(*b*) *DNA synthesis:* Duplicate cover slips were incubated for 2 hr in 199 M containing 10 μCi/ml tritiated thymidine, washed, fixed, and processed for radioautography. Deoxyribonuclease digestion of methanol-fixed material removed the incorporated label completely. Nuclei were easily scored as positive or negative for DNA synthesis.

(*c*) *Radioautography:* Cover slips were treated with 0.3 N trichloroacetic acid (TCA) for 60 min 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 (18).

(*d*) *Surface markers:*

1. *Attachment and ingestion of sensitized erythrocytes:* Heterokaryon preparations were washed twice with 199 and exposed to a suspension containing 2×10^7 sheep red cells per ml and a 1:2000 dilution of rabbit antiserum to sheep red cells. After 15 min incubation at room temperature, red cells had become selectively attached to macrophage surface. The preparations were then washed 4 × and incubated in 199 M for another 15 min at 37°C to allow further ingestion of erythrocytes. The cells were washed in 199, fixed with glutaraldehyde,

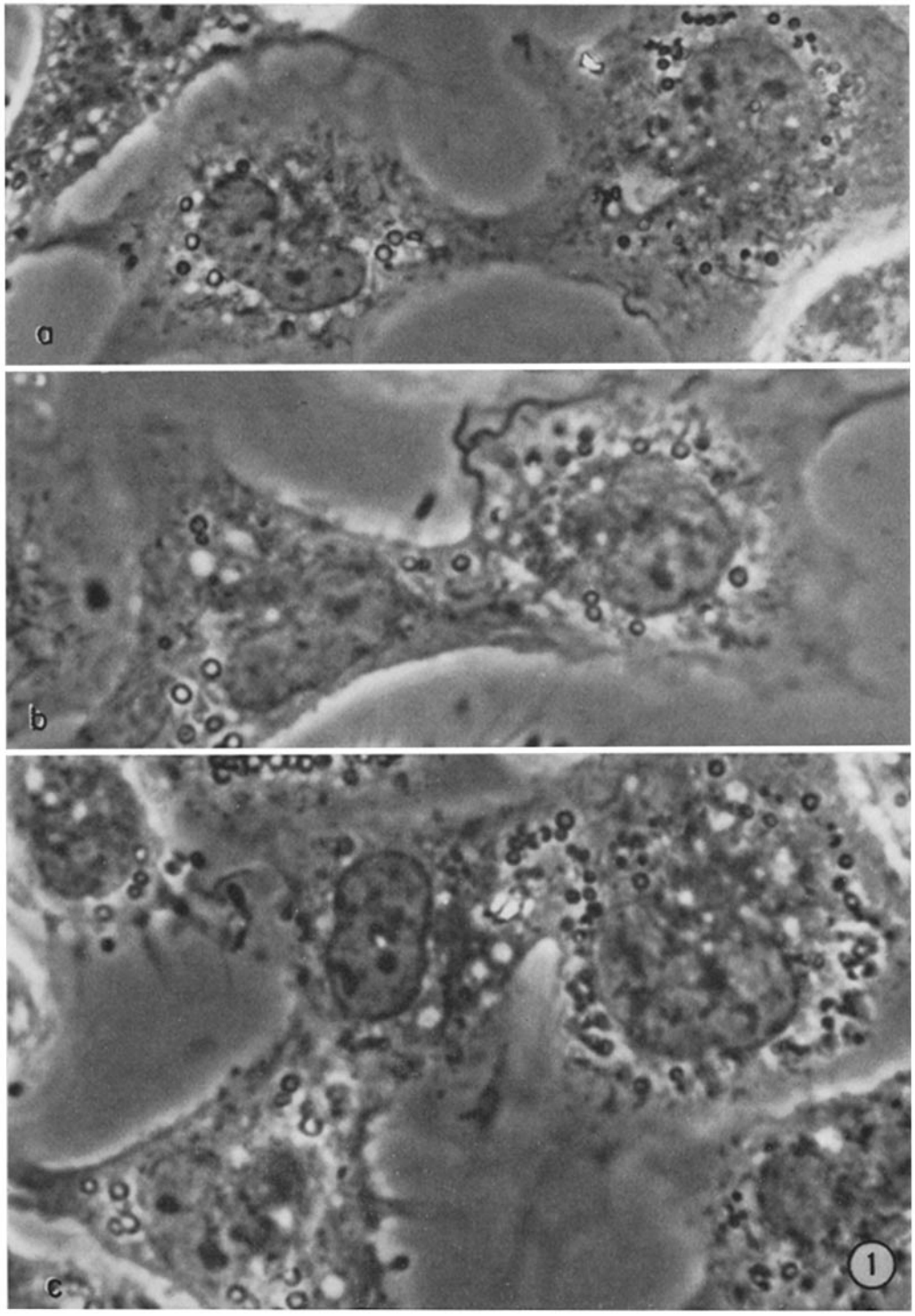
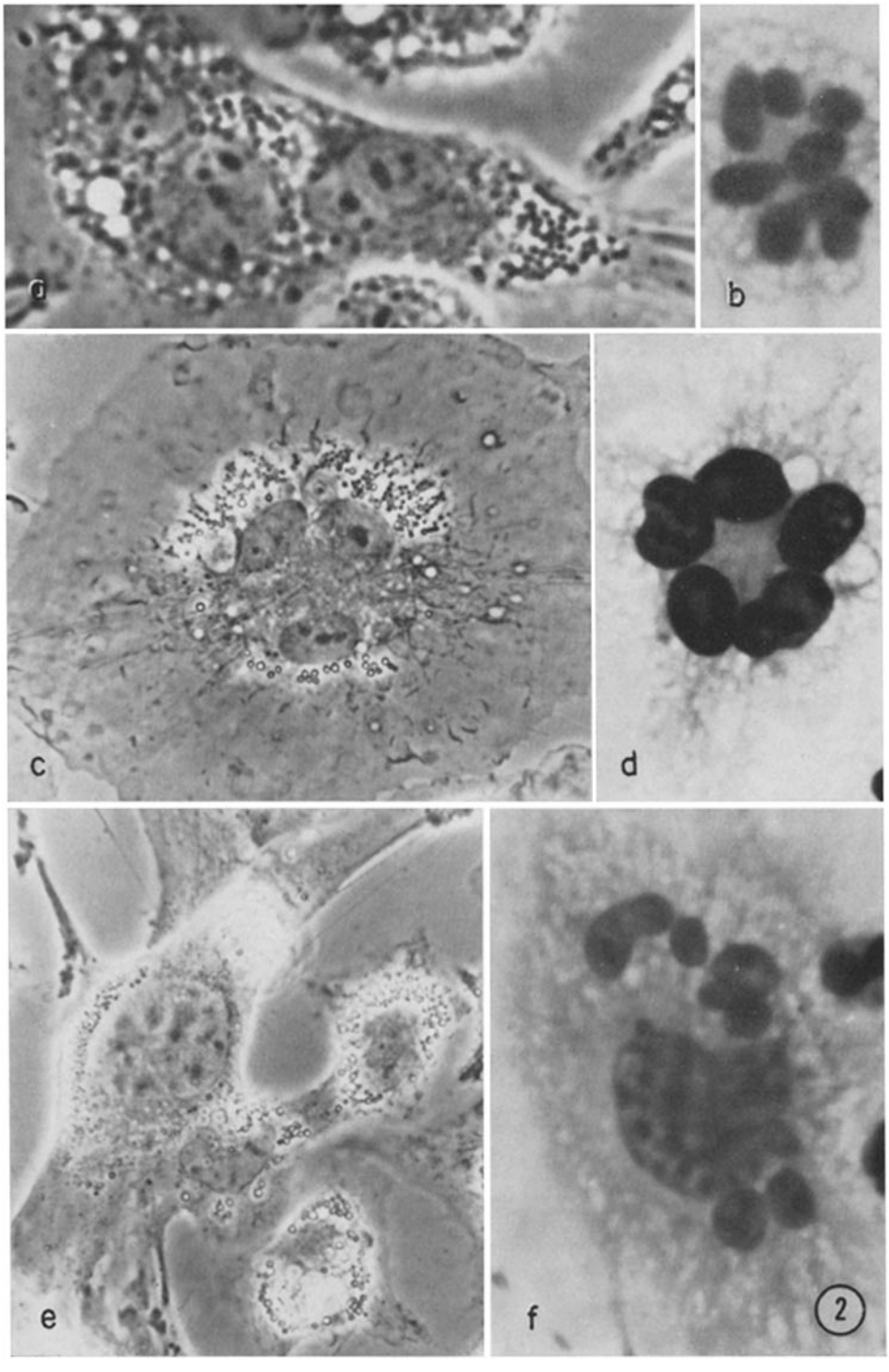


FIG. 1. Early stages in the fusion of mouse macrophages after the addition of Sendai virus. 1.25 % Glutaraldehyde fixation. Phase-contrast. × 2000. (a) 10 min after the addition of virus, a narrow bridge has formed between two adjacent cells. (b) 20 min after the addition of virus, the bridges are wider and contain refractile lipid droplets. (c) 30 min after the addition of virus, the fusion of three macrophages has resulted in the formation of a trinucleate homokaryon.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and stained with a Giemsa stain. Ingested red cells could be distinguished from attached erythrocytes and were counted (7).

2. *ATPase:* The histochemical procedure was based on that of Farquhar and Palade (19). Heterokaryon preparations were fixed with ice cold 1.25% glutaraldehyde in PD for 10 min. Cover slips were washed twice in water and then incubated at 37°C for 30 min 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 min, and treated with a dilute ammonium sulfide solution for 20 sec. 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 (20), care was taken to correlate histochemical investigations 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 cover glasses for 1 day. The cells were washed twice and incubated at 37°C for 20 min 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 (21). The cell monolayer was scraped from the cover slip in normal saline and its protein content measured by the method of Lowry (22). Results were expressed as P_i released per μg protein per min.

The release of P_i was linear under the conditions of assay and the cells remained viable throughout the incubation period. Trichloroacetic 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.

(e) *Acid phosphatase:* Glutaraldehyde-fixed preparations of heterokaryons were examined

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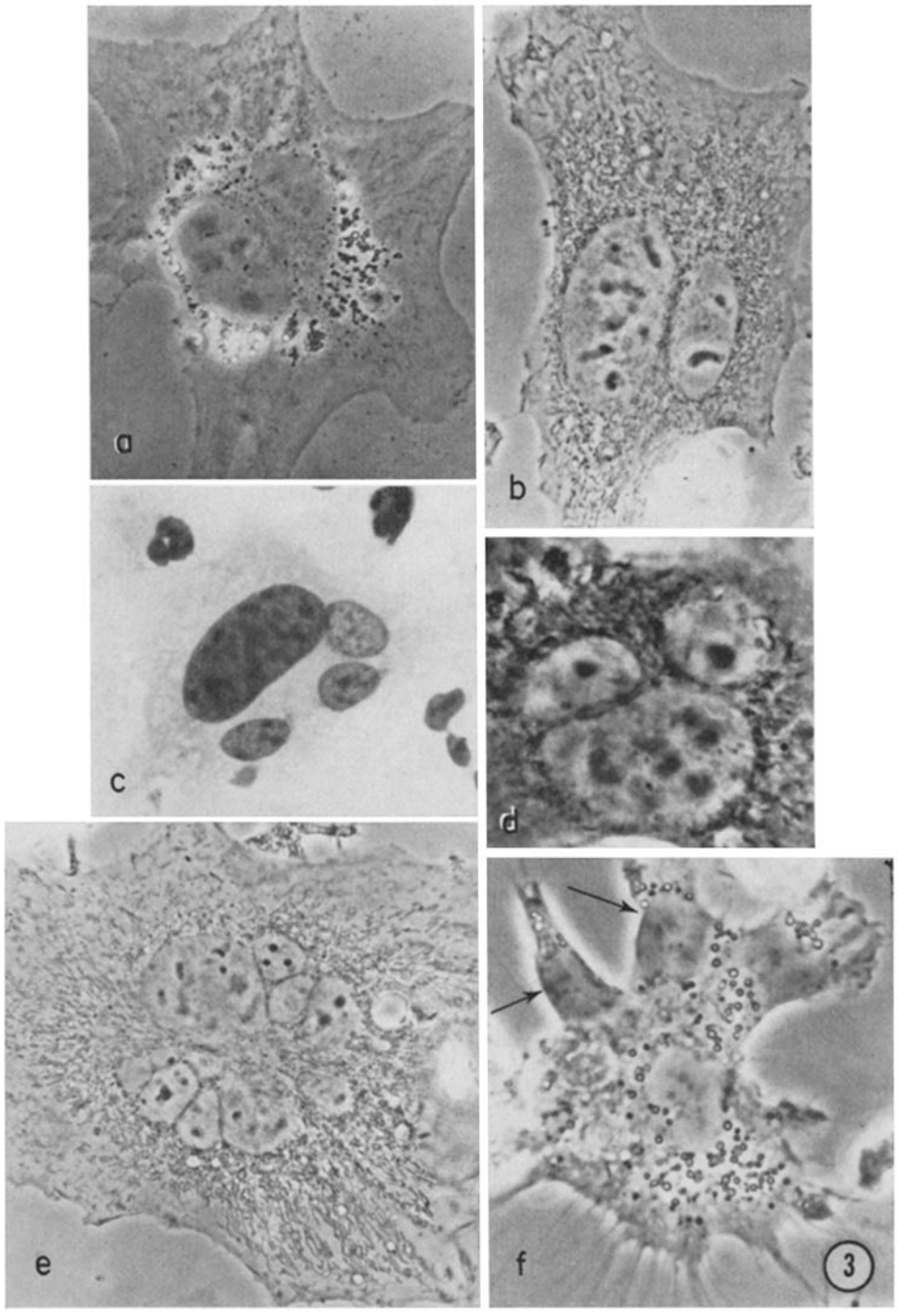
FIG. 2. Later stages in the formation of macrophage homokaryons and macrophage–melanocyte heterokaryons. (a) A trinucleate macrophage homokaryon 2 hr after the start of fusion. The cytoplasm is still unorganized and distinct clusters of lipid droplets remain about each nucleus. Phase-contrast. × 1200. (b) A macrophage homokaryon containing randomly distributed nuclei. May-Grünwald Giemsa stain. × 1000. (c) 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. × 1000. (d) A macrophage homokaryon exhibiting nuclear reorganization. Stained. × 1000. (e) An early stage in the fusion of two macrophages with a melanocyte. Phase-contrast. × 1000. (f) A heterokaryon prior to the reorganization of cellular architecture. Stained. × 1000.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for the presence of acid phosphatase using the histochemical procedure of Cohn and Benson (10). 25 cells of each type were scored at every time point.

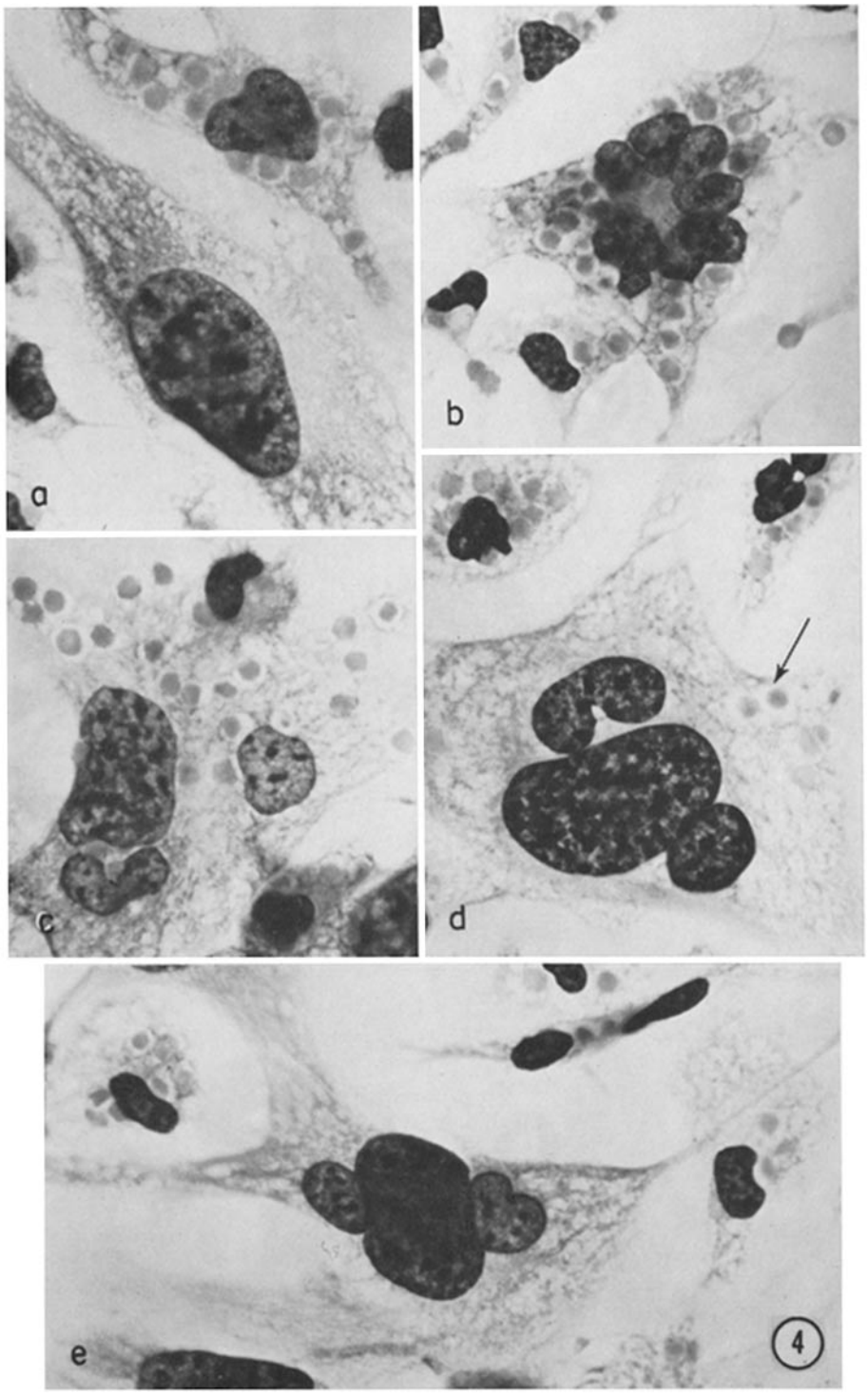
RESULTS

The treatment of cocultivated macrophages and melanocytes with Sendai virus yielded homokaryons and heterokaryons whose properties were studied in detail. These cells underwent changes in morphology and function which could be readily correlated with their composition.

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 1–5 hr, 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. 3 f). 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 melanocyte nuclei were easily distinguished. Melanocyte 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. 3 b and 3 d and can also be seen in Figs. 4 c and 4 d. After several days' cultivation, the heterokaryons resembled melanocytes in morphology. The refractile lipid droplets, of macrophage origin, disappeared from the hetero-

FIG. 3. Examples of fused cells after in vitro cultivation. (a) A 1:1 heterokaryon after reorganization. The lipid droplets are arranged peripherally and the large melanocyte nucleus with its prominent nucleoli is evident. Phase-contrast. × 900. (b) A 1:1 heterokaryon 2 days after fusion. The macrophage nucleus on the right is now enlarged and contains a prominent nucleolus. Phase-contrast. × 1000. (c) A 3:1 heterokaryon after 24 hr of cultivation. The nuclei are now oriented and the three macrophage nuclei are larger than those of adjacent, unfused macrophages. Stained. × 1000. (d) A 2:1 heterokaryon 1 day after fusion. The macrophage nuclei are enlarged and have a single prominent nucleolus. Phase-contrast. × 1000. (e) A large heterokaryon containing one melanocyte nucleus and many macrophage nuclei. Striking reorganization about a common centrosphere region has occurred. Phase-contrast. × 900. (f) A macrophage homokaryon after 5 hr 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. × 1000.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karyons, whereas macrophage homokaryons contained progressively more lipid droplets with prolonged cultivation.

The Fate of Fused Cells.—In order to determine whether macrophage-melanocyte heterokaryons could actually divide, 1:1 heterokaryons, prepared by trypsinization, were observed individually. Unfused melanocytes still present in these preparations usually divided within 1 to 2 days and formed vigorously growing clones. In three separate experiments more than one hundred 1:1 heterokaryons were observed. Of these, 30 to 80%, depending on the particular experiment, yielded pairs of descendants. This usually occurred 2–4 days after fusion and resulted in cells morphologically indistinguishable from normal melanocytes. In some instances heterokaryons were seen in mitosis. Since most heterokaryons eventually entered mitosis, there were none left as such by the 7th day after fusion.

Fewer cells went into a second division and in only one case could a third division be traced directly. At a time when the unfused melanocytes had formed colonies of over 100 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 melanocytes.

Shortly after the formation of heterokaryons, the dextran sulfate vacuoles of macrophage origin were randomly distributed in the common cytoplasm. On the 1st 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 melanocytes, 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 2nd day after fusion. Their progeny were morphologically indistinguishable from normal, unfused melanocytes except for their sluggish proliferation.

FIG. 4. The phagocytosis of opsonized sheep erythrocytes by homokaryons, heterokaryons, and unfused macrophages. Stained. × 1000.(a) An unfused macrophage has ingested many erythrocytes, whereas an adjacent melanocyte contains none. (b) Extensive red cell phagocytosis by a multinucleated homokaryon. (c) A 2:1 heterokaryon 2 hr after fusion. The macrophage nuclei have already enlarged and the cell has phagocytized many erythrocytes. (d) A 2:1 heterokaryon 12 hr after fusion. Only two erythrocytes have been ingested. Adjacent, unfused macrophages with smaller nuclei contain many erythrocytes. (e) A 2:1 heterokaryon has failed to ingest erythrocytes 24 hr after fusion.

The Stimulation of RNA and DNA Synthesis in Macrophage Nuclei of Heterokaryons.—

RNA synthesis: Unfused macrophages were able to make RNA, but after fusion with a melanocyte there was a 4- to 10-fold increase in RNA synthesis by the macrophage nuclei, as judged by grain counts. This stimulation could be detected by radioautography 2 hr after fusion and developed fully over the next few hours. RNA synthesis was increased irrespective of the number of macrophage nuclei present in a heterokaryon. A lesser stimulation, two- to three-fold, could be found in macrophage homokaryons.

Radioautography showed an increased number of grains over the enlarged macrophage nucleoli, as well as over the rest of the macrophage nucleus.

DNA synthesis: Macrophages and their homokaryons did not incorporate radioactive thymidine into DNA at any time. The macrophage nuclei of heterokaryons were specifically stimulated to make DNA. Heterokaryons with several macrophage nuclei often showed synchronous labeling of all the nuclei and there was no evidence that the macrophage could inhibit DNA synthesis in the melanocyte nucleus.

Although 50–80% of melanocytes were making DNA at the time of fusion, there was a lag of at least 3 hr before the macrophage nuclei started to make DNA. During the following 4 hr, more than 80% of the macrophage nuclei in heterokaryons became labeled in those cells where the melanocyte nucleus was also labeled.

The Fate of Macrophage Markers after Fusion.—Major differences exist in the specialized functions which the macrophage and melanocyte 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.

Phagocytosis.—The ability of the macrophage to ingest particles coated with 7S antibody is an example of such a specific 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 exposed to a rabbit antiserum and their uptake by fused cells studied quantitatively. Experiments assaying phagocytic function are illustrated in Fig. 4 and Figs. 5–8 and the results of a typical experiment are listed in Table I. 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 (See Fig. 5. The crosses represent the mean number of red cells ingested; the bars, twice the standard deviation of the mean). The phagocytic activity of heterokaryons at different times after fusion is illustrated in Fig. 6. Each type of heterokaryon was compared with homokaryons containing the same number of macrophage nuclei (Table I). Heterokaryons, studied soon after fusion, ingested as many red cells as their controls, but

this ability disappeared within a few days (Fig. 4). 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. 7.

30 cells were scored on four replicate cover slips at each time point. The circles represent the mean number of red blood cells ingested by 30 heterokaryons on a particular cover slip, the crosses represent the mean for each group of four cover slips.

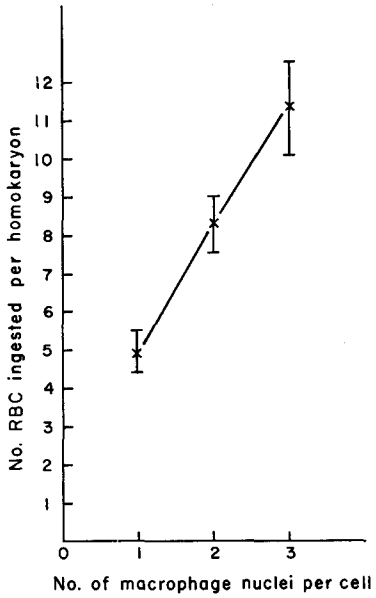


FIG. 5. The ingestion of sensitized erythrocytes by macrophages and macrophage homokaryons.

It can be seen that the decay curve is exponential, with a half life of 2.7 hr.

To determine whether the failure of older heterokaryons to ingest red cells was due to the failure of attachment or the ingestion process itself, heterokaryons of different ages were exposed to the red cells for 15 min at room temperature, washed 4 ×, and fixed immediately. Whereas almost all macrophages had three or more attached red cells, none were present on the melanocytes. 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.

To gain further insight into the mechanism of disappearance of the macro-

TABLE I

Phagocytosis by Macrophage Homokaryons and Macrophage–Melanocyte Heterokaryons

Time after fusion	Type of cell (macrophage: melanocyte)	No. of cells scored	Mean No. RBC ingested per cell	SD of the mean	Control*
					%
4 hr	1:0	30	2.8	0.31	
	2:0	25	7.3	0.57	
	3:0	11	9.9	1.3	
	1:1	39	2.5	0.52	89
	2:1	18	6.7	1.2	92
	3:1	9	8.9	1.6	90
9 hr	1:0	31	3.5	0.33	
	2:0	30	6.8	0.65	
	3:0	22	9.6	1.2	
	1:1	52	1.8	0.44	51
	2:1	23	5.1	1.1	75
	3:1	13	10.4	2.5	100
1 day	1:0	30	3.1	0.36	
	2:0	26	4.1	0.45	
	3:0	25	7.6	0.59	
	1:1	81	0.65	0.098	21
	2:1	34	2.3	0.53	56
	3:1	16	5.2	0.67	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	0.24	13
	2:1	31	3.3	0.76	26
	3:1	12	8.5	1.8	39
4 days	1:0	40	7.5	0.62	
	2:0	43	8.7	0.71	
	3:0	40	11.1	0.96	
	4:0	30	15.1	1.5	
	1:1	68	0.29	0.089	3.8
	2:1	46	0.91	0.34	11
	3:1	9	1.7	0.76	15
5 days	1:0	10	6.4	1.0	
	2:0	10	9.4	0.95	
	3:0	10	16.7	2.2	
	1:1	44	0.34	0.13	5.3
	2:1	22	0.82	0.35	8.8
	3:1	13	0.70	0.26	4.2

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

phage surface marker, heterokaryons were cultivated under conditions which stimulated pinocytosis and membrane interiorization.

Replicate preparations were fused in the usual way by the addition of virus for 1 hr at 37°C, washed twice, and then divided into two groups. One group was cultivated in 199, plus 4%

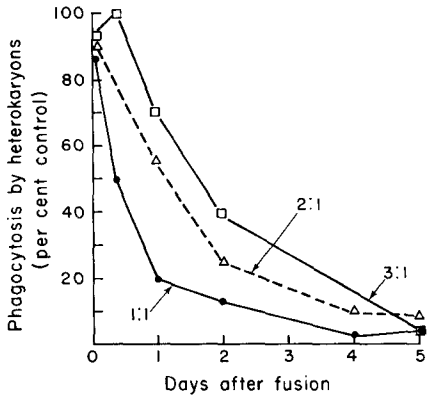


FIG. 6. Phagocytosis by macrophage–melanocyte heterokaryons. Macrophage homokaryons containing the same number of macrophage nuclei served as controls.

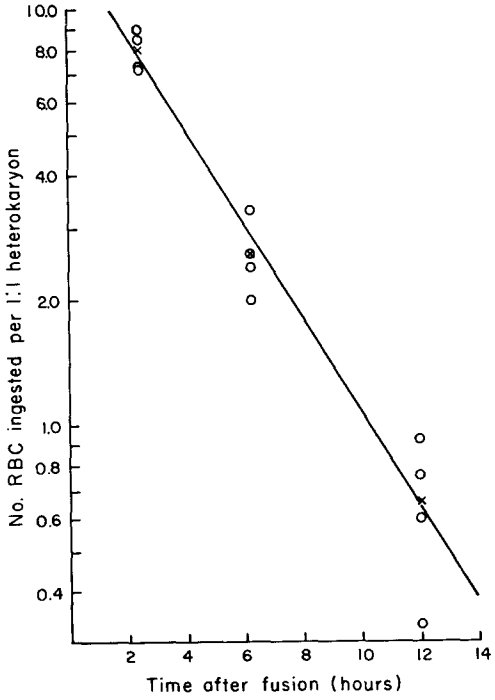


FIG. 7. The early loss of phagocytic function in 1:1 heterokaryons.

newborn calf serum, the other in 199 plus 40% newborn calf serum. Replicate preparations were assayed for phagocytosis at various times (Fig. 8). The circles and squares represent the mean number of red cells ingested by 30 heterokaryons on each coverslip, the crosses the mean for each group of 4 cover slips.

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 for 1 wk. The loss of

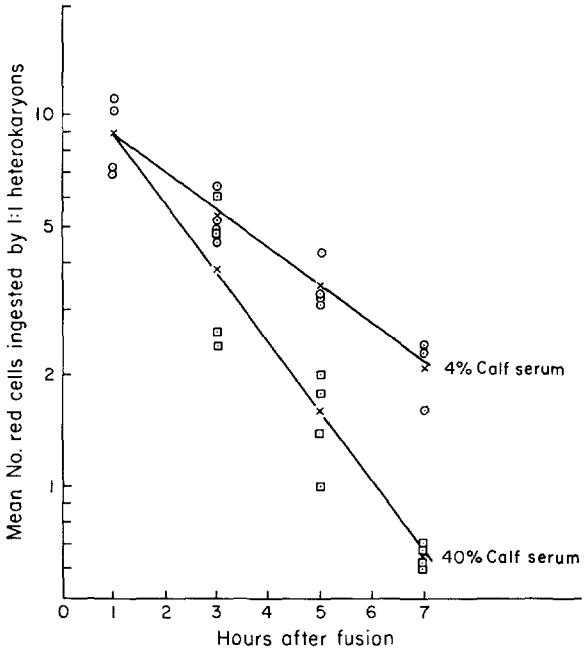


FIG. 8. The effect of serum concentration on red cell uptake by 1:1 heterokaryons.

phagocytic function from 1:1 heterokaryons was almost complete by the 2nd day after fusion and did not return by the 7th day. These heterokaryons have very prominent macrophage nucleoli from the 1st 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. The loss of phagocytic ability was virtually complete before the first division and none of the daughter cells, which were identified by the presence of dextran sulfate, recovered this property over the next week.

ATPase Activity.—Intact macrophages have 30 times more surface ATPase activity than melanocytes and this difference is sufficient to be useful as a cytochemical marker. It was possible to follow the presence, as well as the dis-

tribution 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

TABLE II

The Liberation of Pi from Different Substrates by Macrophage Surface ATPase

Substrate	μmoles P_i/μg protein per min	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
d-AMP 5′	0	0
Cyclic AMP	0	0
Glucose-6-phosphate	0	0
Na α-naphthyl acid phosphate	0	0
Disodium β-glycerophosphate	0	0

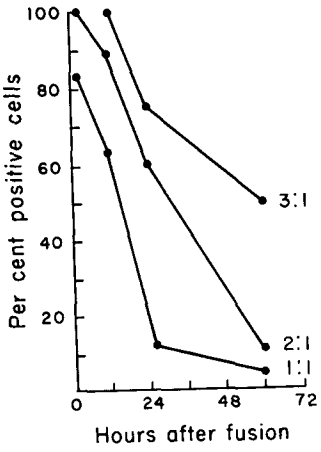
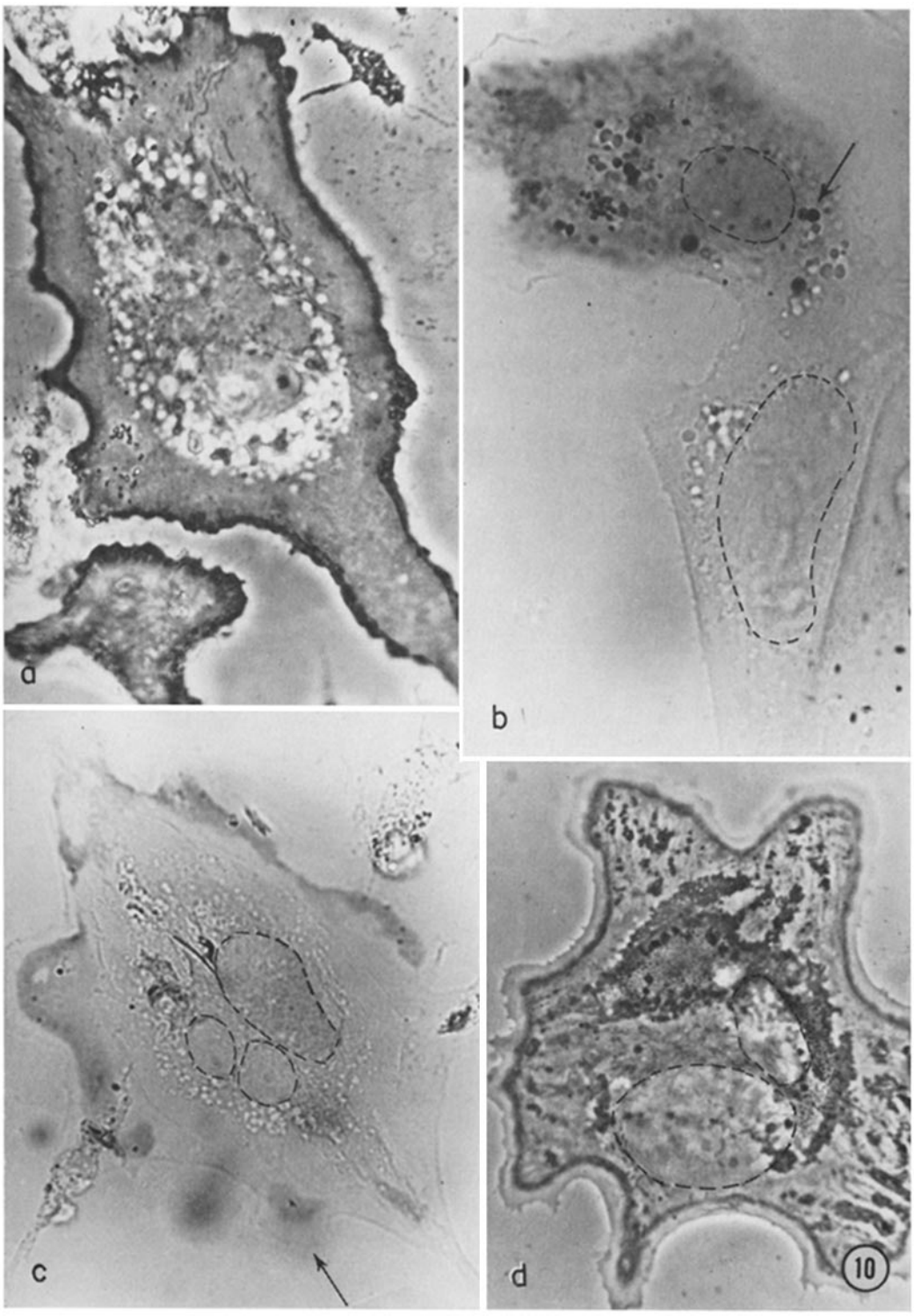


FIG. 9. The loss of cytochemically demonstrable ATPase activity in heterokaryons.

in Table II. The enzyme is specific for nucleoside 5′ phosphates and ATP is hydrolyzed most actively. The rather active hydrolysis of ADP and, to a lesser extent AMP5′, suggests that stepwise P_i release could be occurring during the incubation procedure. The enzyme showed an absolute requirement


a
b
c
d
10

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 melanocytes showed no reaction product under the light microscope. Some peripheral vesicles, 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.

The fate of macrophage ATPase in heterokaryons is shown in Fig. 9. 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. 10. In the first 12 hr after fusion, when heterokaryons were still staining, the reaction product often involved only a "patch" of the total cell surface. Other heterokaryons, including 1:1 cells, showed a fainter, more diffuse surface distribution or a margin about the cell. Patches were still seen in heterokaryons after 1 day, especially in 3:1 heterokaryons, but the reaction product was usually faint and diffuse by 36 hr.

DISCUSSION

The cultivation of peritoneal cells for 24 hr prior to the addition of virus resulted in a uniform population of macrophages which were evenly spread 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 (23).

When cells are fused with virus, each partner contributes a nucleus, a va-

FIG. 10. The cytochemical demonstration of ATPase activity in homokaryons and heterokaryons. Fixed in 1.25% glutaraldehyde prior to incubation with substrate. (a) A macrophage homokaryon exhibiting intense reaction product about the cell periphery. Phase-contrast. × 1000. (b) 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 (arrow) are also stained. In contrast, the melanocyte surface and vesicles are free of reaction product. Bright field. × 1000. (c) A 2:1 heterokaryon 3 hr 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. × 800. (d) A 1:1 heterokaryon 8 hr after fusion. A fine reaction product outlines the cell periphery. Coarser aggregates are seen over the cell surface. Bright field. × 1200.

riety 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–melanocyte 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 on the formation of syncytia (24). The importance of cell origin on the fate of organelles becomes critical only in the later events after fusion.

Macrophage homokaryons remain viable for many days after fusion. 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 (25).

In contrast, when a macrophage is fused with a melanocyte, it undergoes drastic changes in its behavior. The macrophage nucleus swells and its nucleoli become prominent. RNA synthesis is stimulated and DNA synthesis is initiated. Distinctive macrophage surface and cytoplasmic markers can be demonstrated in recently fused heterokaryons, but become undetectable within the next day or two. Macrophage organelles like lysosomes and lipid droplets can also be identified in fused cells. Their disappearance from heterokaryons contrasts strikingly with their accumulation 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.

A more quantitative analysis was performed of the fate of macrophage markers at the surface of the heterokaryons. The mouse macrophage has surface receptor sites for antibody-coated sheep red cells which probably play an important part in the early stages of the phagocytic process (12). Although the phagocytic marker used is a complex one, it is clear that the failure of older heterokaryons to ingest red cells was associated with the failure of red cells to become attached to the heterokaryon surface. This suggested that the appropriate surface receptors had disappeared or were altered in some way which prevented their detection. The loss of phagocytic function proceeded steadily, even when several macrophages had fused with a single melanocyte. A possible explanation for this loss of function lies in the continuous flow of macrophage membrane into the interior of the heterokaryon without renewal at the cell surface. Such a process of continuous membrane flow into the cell would be expected to be influenced by pinocytic activity. The ability of high concentration of serum, a known stimulus for pinocytosis, to accelerate the loss of the phagocytic marker, can be regarded as further evidence for such a mechanism.

However, other explanations which involve reorganization at the cell surface and result in a masking or dilution of macrophage receptors cannot be excluded.

The loss of detectable surface ATPase from heterokaryons is compatible with the suggested mechanism of interiorization without replacement. The apparent change in distribution of this marker from discrete regions of activity to a more diffuse and fainter type of reaction suggests that a mixing process might be taking place at the cell surface at the same time. Antigens have been studied as a surface marker in other heterokaryons and their initial behavior in those studies supports the present theory of clearance from the surface of the heterokaryon, as well as the occurrence of a mixing process (26, 27).

There was no recovery of macrophage-specific functions in the 7-day period after the formation of macrophage–melanocyte heterokaryons. Harris and coworkers demonstrated that heterokaryons containing chick red cell nuclei regain some chick-specific markers when the chick nucleoli mature several days after fusion (27). Since the macrophage nucleoli in heterokaryons become prominent and label heavily with uridine within hours of fusion, it is unlikely that the failure to regain macrophage-specific markers within a week is due to a similar mechanism.

3 hr after fusion with melanocytes, DNA synthesis was stimulated in previously inactive macrophage nuclei. Even when a single melanocyte was fused with several macrophages, the outcome of the interaction was the same—the melanocyte function always predominated, a finding which agrees with the earlier observations of Harris and coworkers in similar systems (5). Although many 1:1 heterokaryons divided at least once, it was clear that the offspring of these cells had a reduced ability to proliferate.

Experiments to elucidate the mechanism by which DNA synthesis is activated in the macrophage nucleus will be reported in a subsequent paper.

SUMMARY

High yields of mouse macrophage–melanocyte heterokaryons and macrophage–macrophage homokaryons were obtained through the 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 melanocyte-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. 3 hr after fusion, a wave of DNA synthesis took place in the previously dormant macrophage nuclei.

The fate of typical macrophage markers was examined in both heterokaryons

and homokaryons. Macrophage homokaryons continued to exhibit active phagocytosis of sensitized erythrocytes, whereas this capacity was lost irreversibly in heterokaryons. The loss of phagocytic activity of heterokaryons occurred at an exponential rate and was accelerated by high concentrations of calf serum.

Another macrophage surface marker, a divalent cation-dependent adenosine triphosphatase (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.

Acid phosphatase-positive secondary lysosomes and refractile lipid droplets disappeared from heterokaryons but continued to accumulate in macrophage homokaryons.

These observations indicate that typical macrophage properties cease to be expressed in heterokaryons, and melanocyte functions presumably predominate in heterokaryons and hybrids.

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