

THE PREPARATION AND PROPERTIES OF MACROPHAGE-L CELL HYBRIDS*

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(Received for publication 24 June 1971)

Cell hybridization provides a useful tool to study cellular differentiation. Differentiated cells can be fused with other cells by means of Sendai virus; the fate of specialized cell properties in the resulting hybrids may provide new insight into the control of gene expression in animal cells (1, 2).

For the present studies we chose to fuse mouse peritoneal macrophages with LMTK⁻ cells, a strain of mouse fibroblasts. The plasma membrane of the macrophage has specific receptors which enable the cell to bind IgG or complement-coated sheep red cells and is also rich in a divalent cation-dependent adenosine triphosphatase (ATPase)¹ activity; LMTK⁻ cells lack these macrophage membrane markers (3-6). Macrophages were obtained from inbred DBA/2 mice so that two other genetic markers could be used to characterize hybrid cells, the H-2 antigen, and phosphoglucose isomerase (PGI) isozymes (7).

In the present paper we describe the preparation of macrophage-L cell hybrids and show that these hybrids express various macrophage-derived membrane properties to a different degree.

Materials and Methods

Cells.—Female DBA/2 mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. Macrophage cultures were established as described previously, 1×10^6 cells/22 mm² cover slip (6). After 1 day's cultivation the monolayers were trypsinized to remove any fibroblasts. The monolayer was washed twice in phosphate-buffered saline without Ca⁺⁺ and Mg⁺⁺ incubated with 0.25% trypsin-0.05% Versene (TV) solution for 10 min at 37°C, and then washed well with Eagle's medium before further cultivation. LMTK⁻ cells were provided by Dr. F. Ruddle, Yale University, New Haven.

Media.—Parental cells were cultured in medium E: Eagle's medium, 10 or 20% newborn

* This work was partially supported by Grant AI 07012 from the National Institutes of Health.

‡ Special Fellows of the Leukemia Society of America, Inc.

¹ *Abbreviations used in this paper:* ATPase, adenosine triphosphatase; Fc receptor, receptor for IgG-coated SRBC; HAT, selection medium containing hypoxanthine, aminopterin, and thymidine; LD, LMTK⁻ × DBA/2 macrophage hybrid; PGI, phosphoglucose isomerase; SRBC, sheep red blood cells.

calf serum, sodium penicillin G (50 units/ml), and streptomycin sulfate (10 $\mu\text{g}/\text{ml}$). Tylocine, 60 $\mu\text{g}/\text{ml}$, or Aureomycin 50 $\mu\text{g}/\text{ml}$ (Grand Island Biological Co., Grand Island, N. Y.) were added on occasion.

Hybrid cells were grown in HAT medium: medium E, supplemented with 1×10^{-4} M

TABLE I
Isolation of Macrophage-LMTK⁻ Hybrids

Fusion =	
0	
	<i>days</i>
-2	DBA/2 macrophages in culture ($1 \times 10^6/22 \text{ mm}^2$ cover slip)
-1	Monolayer trypsinized
0	LMTK ⁻ cells added ($1 \times 10^6/35 \text{ mm}$ dish) fused with 1000 hag ^u . ultraviolet Sendai
1	Trypsinized. Replated in HAT. HAT changed every 3-5 days
13	Colonies detected
18-33	Colonies isolated
	LD ₃ (clone) LD ₄ (mass culture) LD ₅ (clone) LD ₇ (clone)
25-90	Each grown to mass culture and propagated in vitro
	Aliquot frozen Karyotype PGI isoenzymes Surface properties assayed: H-2, ATPase, Fc, and complement receptors

hypoxanthine, 1×10^{-5} M aminopterin (K & K Laboratories, Inc., Plainview, N. Y.), and 1.6×10^{-5} M thymidine. Glycine (4×10^{-4} M) is present in medium E.

Cell Hybridization.—Table I summarizes the production, isolation, and study of macrophage-L cell hybrids. Confluent LMTK⁻ cells were trypsinized and added to 2-day old macrophage monolayers. After 4 hr, ultraviolet-inactivated Sendai virus was added for 1 hr. The

fused preparations were diluted by trypsinization 1 day later and placed in HAT. Colonies were detected by 13 days and three clones (LD₃, LD₅, and LD₇) and one mass culture (LD₄) successfully grown and carried in culture for 3 months (LD = LMTK⁻ × DBA/2 macrophage hybrid). Only one clone was picked per dish, using the cloning cylinder method of Puck et al. (8). Cell hybrids were subcultured by trypsinization at a one-third/one-fifth dilution or stored at -70°C in medium containing 10% glycerol.

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

Chromosome Studies.—Performed 27–53 days after fusion. Hybrid LD₃ and LD₅ 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 (Falcon Plastics, Los Angeles, Calif.) and treated with Velban (Eli Lilly and Co., Indianapolis, Ind.), 0.025 μg/ml, for 3–4 hr. The cells were collected by trypsinization and swollen by exposure to 0.075 M KCl for 15 min at 37°C. Preparations were fixed 1:3 acetic acid + methanol, chromosome spreads prepared by air drying, and stained with 2% aceto-orcein (Grand Island Biological Co.).

Phosphoglucose Isomerase (EC 5.3.1.9) Phenotypes.—Determined by starch gel electrophoresis, with minor modifications of the method of DeLorenzo and Ruddle (9). Fructose-6-phosphate was obtained from Boehringer Mannheim Corp., New York.

Homogenates were prepared from 1–1.5 × 10⁷ cells obtained by trypsinization or scraping, in the case of macrophage cultures. The cells were washed in phosphate-buffered saline and then disrupted in 0.01 M phosphate buffer, pH 7.0, with the aid of a teflon homogenizer. The homogenates were centrifuged for 60 min in a Lourdes centrifuge (Lourdes Instrument Corp., Old Bethpage, N. Y.) at 30,000 g and the clear supernatant frozen at -20°C.

Surface Properties.—

(a) *H-2 antigens:* Examined by mixed hemadsorption assay (10). Isoantisera, described in Table III, were donated by Dr. E. Boyse of the Sloan-Kettering Institute, New York. Normal mouse serum was obtained by cardiac puncture of NCSR female mice from the Rockefeller colony and used in control studies.

A 1% suspension of sheep red blood cells (SRBC) in 199 was coated at 37° for 30 min 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 anti-mouse IgG serum (Microbiological Associates, Inc., Bethesda, Md.). These indicator red cells were washed twice in 199 and finally resuspended as a 1/4% cell suspension in 199.

Cells were grown on cover slips for 1–4 days before assay. Preparations were incubated for 1 hr at 4°C with serial dilutions of iso-antiserum in 199. The cover slips were then washed twice in 199 and incubated with two drops of the indicator red cell suspension for 1 hr at 4°C. The cells were washed vigorously, four times in 199, and then examined by phase-contrast 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:* Performed on viable nonconfluent hybrid or L cells grown for 1–4 days in 60-mm Falcon plastic dishes. DBA/2 macrophages were grown on 22-mm² cover slips. The biochemical assay used has been described previously (7). For the present studies, the incubation mixture contained both mono- and divalent cations. Assays were done in triplicate and the results expressed as micromolar P_i released, per microgram of protein, in 30 min.

(c) *Receptor for IgG-coated SRBC (Fc receptor):* The Fc receptor was assayed by a slight modification of the procedure used previously (6). Sheep red cells were coated with a 1/2000 dilution of rabbit antiserum and used immediately. Cover slip preparations were washed twice in 199, incubated for 15–90 min with antibody-coated red cells at room temperature or 37°C, washed well, and then examined. Controls were incubated with a similar SRBC suspension which had not been coated with antibody.

(d) *Receptor for complement*: The macrophage receptor for complement was assayed on cover slips by the procedure of Lay and Nussenzweig (4). A 1% SRBC suspension was coated with a 1/10,000 dilution of rabbit amboceptor (Behring Diagnostics Inc., Woodbury, N. Y.) which had been previously heat inactivated at 56°C for 30 min. A 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 at (56°C for 30 min). Cover slips were washed twice in 199, incubated with one drop of a 0.5% suspension of the indicator cells for 30 min at 4°C, washed well, and examined.

RESULTS

General Considerations

Since macrophages do not divide in culture it was only necessary to devise a half-selection system to isolate hybrids (7, 11). LMTK⁻ cells are killed by HAT medium; the macrophage gene for thymidine kinase becomes activated after fusion with LMTK⁻ cells so that only hybrid cells proliferate in HAT medium.

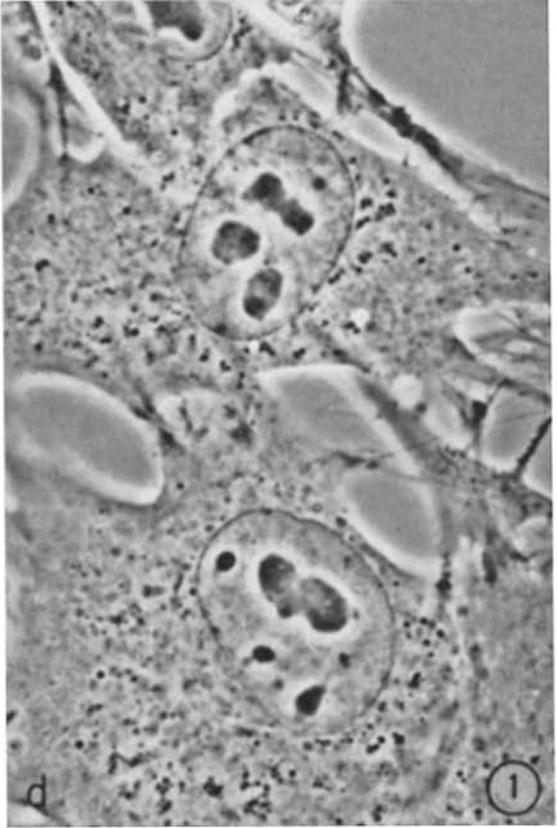
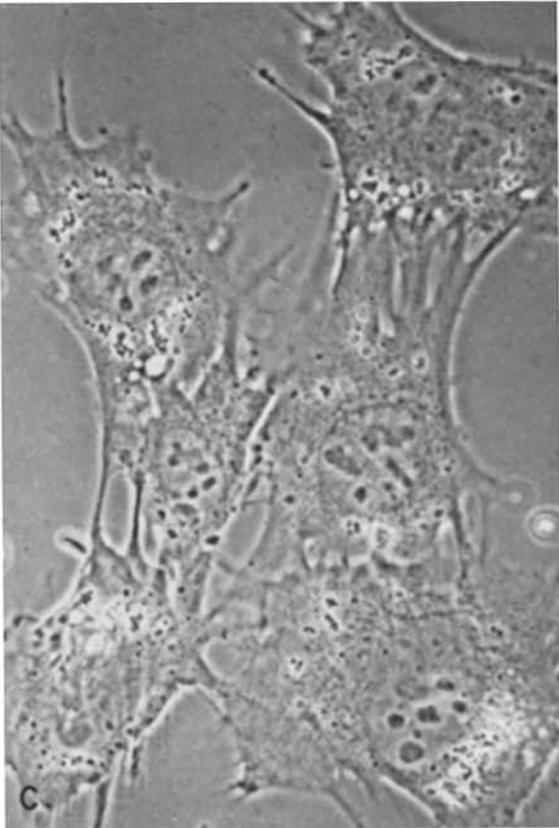
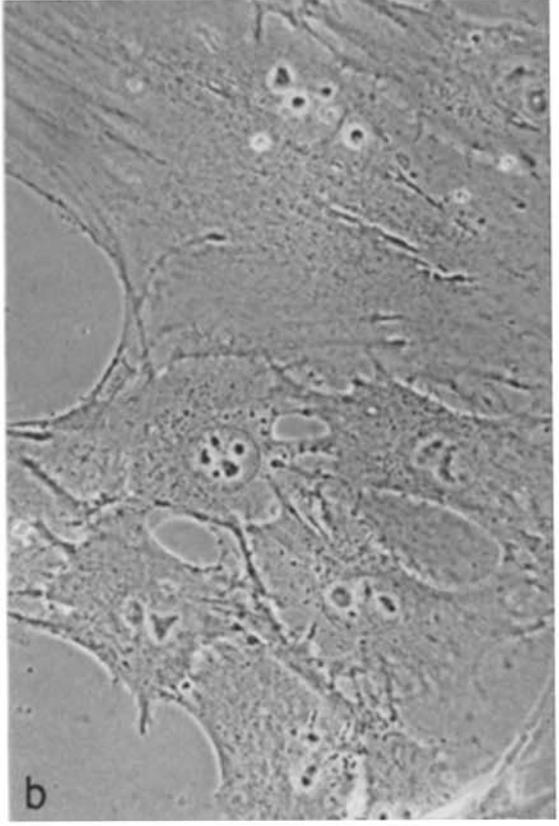
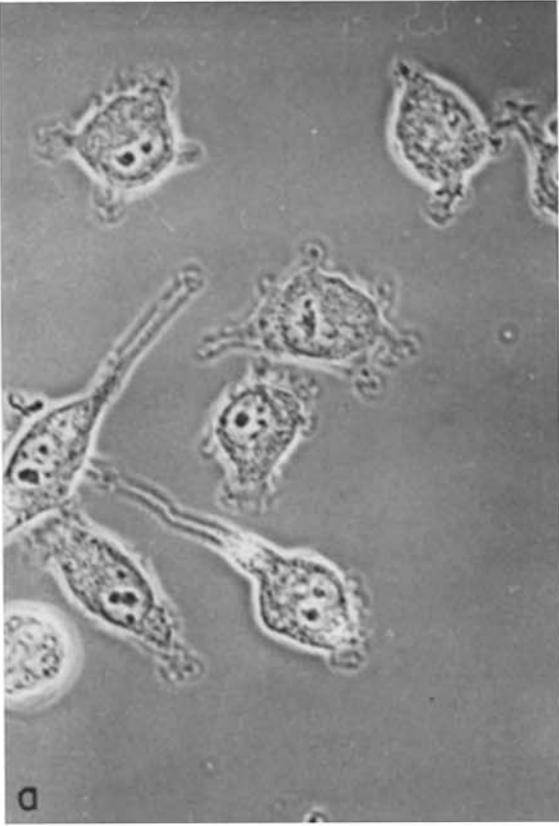
Two precautions were taken to isolate true macrophage hybrids rather than LMTK⁻ × DBA peritoneal fibroblast hybrids. Cell cultures were obtained from animals with uninflamed peritoneal cavities so that very few contaminant fibroblasts should be present. Macrophages and peritoneal fibroblasts also react differently to trypsinization; macrophages remain adherent to glass whereas fibroblasts become detached. Macrophage cultures were therefore trypsinized before fusion to remove contaminant fibroblasts.

The general properties and karyotype of the hybrids were next investigated
Morphology and Growth.—LMTK⁻ cells spread very slowly on glass or plastic (Fig. 1 *a*) and double every 19 hr during the phase of exponential growth. At confluence the cell monolayer has a characteristic cobblestone appearance (Fig. 2), 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 show no contact inhibition of movement.

Hybrid cells show features of both parents (Figs. 1 *b-d*, 2 *b*). Their nuclei are larger than those of either parent cell. Different hybrid clones resemble one another and all spread extensively within an hour of trypsinization. The hybrid cells often grow in clusters and frequently overlap one another (Fig. 1 *d*). They double every 36 hr during exponential growth. At confluence the hybrids appear more fibroblastic, without heaping up.

FIGS. 1 *a-d*. L cells and hybrids cultivated on glass for 1 day. Phase contrast. (*a*) LMTK⁻ cells are poorly spread. × 500. (*b*) Hybrid LD₃ cells are flat and well spread. The nuclei are larger than in the L cells. × 500. (*c*) Hybrid LD₃. Well-spread cells with multiple small pseudopodia. × 625. (*d*) Hybrid LD₃. Cell processes overlap one another. × 1250.



Chromosome Studies.—LMTK⁻ cells have a mean number of 46 chromosomes, which include 11–13 metacentric marker chromosomes (Table II, Fig. 3). The karyotype of DBA/2 macrophages was not examined, but should consist of the normal diploid mouse complement of 40 chromosomes, all of which are acrocentric (12). 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 from the fusion of one LMTK⁻ cell with one macrophage.

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.

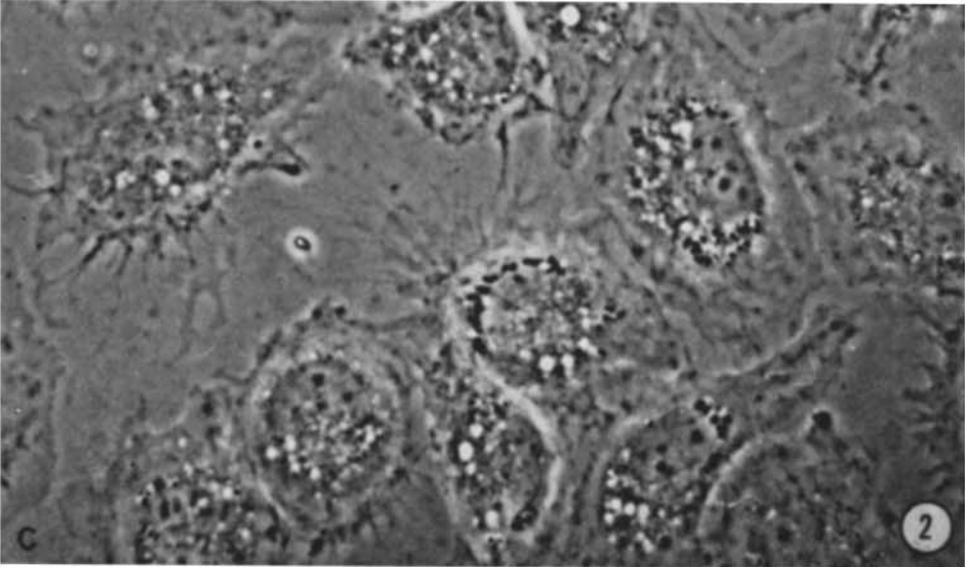
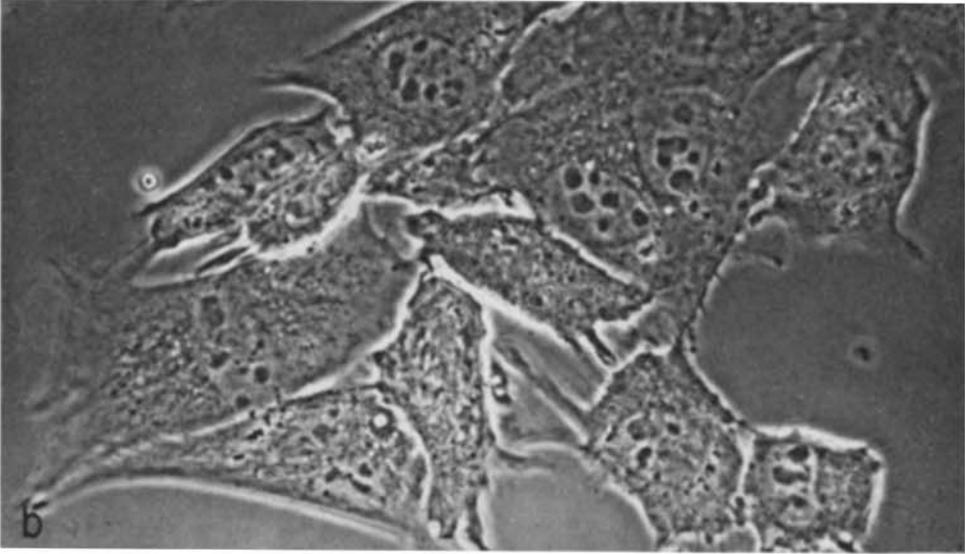
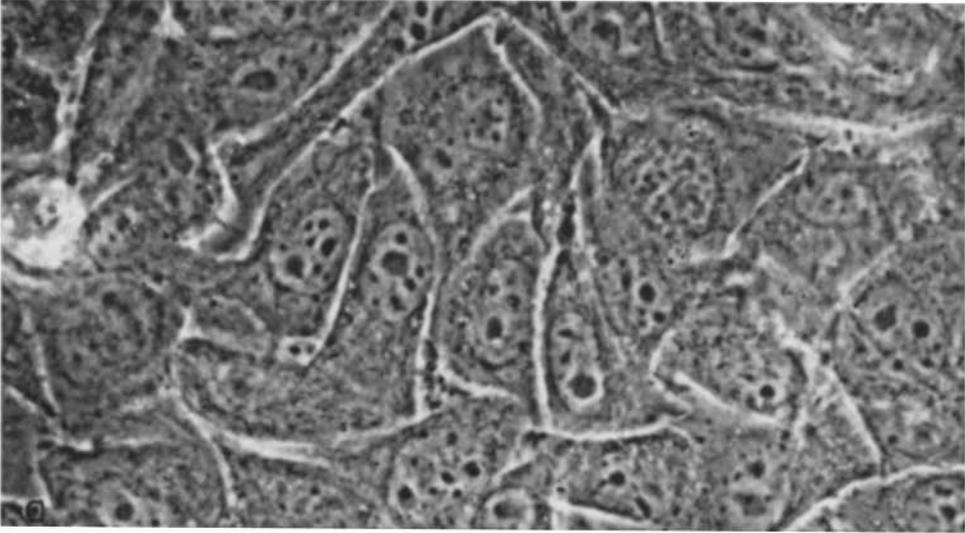
Surface Properties.—

(a) *H-2:* The results of the mixed hemadsorption assay are given in Table III. L cells, which originate from a C₃H mouse, show only H-2k activity (titer 1/3000), whereas the macrophages from DBA/2 animals have H-2d antigens. They were not examined in the present studies since the indicator cells used here would react with the phagocytic receptor, irrespective of H-2 genotype. If the exposed Fc fragment on the indicator red cells is removed by treating the rabbit IgG with pepsin, H-2 activity of the appropriate genotype can be demonstrated in macrophages (unpublished observations).

All the hybrids showed both H-2k and H-2d activity. The titer varied somewhat, e.g., LD₃ cells had a higher H-2k titer than the other hybrid cells (1/10,000 vs. 1/3000). The H-2d titer was 1/3000–1/1000. The difference between d and k corresponds with the different titer which these two antisera display in a cytotoxic assay with lymph node cells. At higher antiserum concentrations all reactions were diminished or 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 IV. 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%. Addition of ouabain had no effect on hybrid ATPase activity.

FIGS. 2 *a-c.* Morphology of parent and hybrid cells at or near confluence. Phase contrast. (a) LMTK⁻ cells show a cobblestone appearance. $\times 625$. (b) Hybrid LD₇. The cells show more spreading, but also resemble L cell growth pattern. $\times 500$. (c) Macrophages are well spread, with multiple small pseudopodia. $\times 625$.



(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 2 hr in the

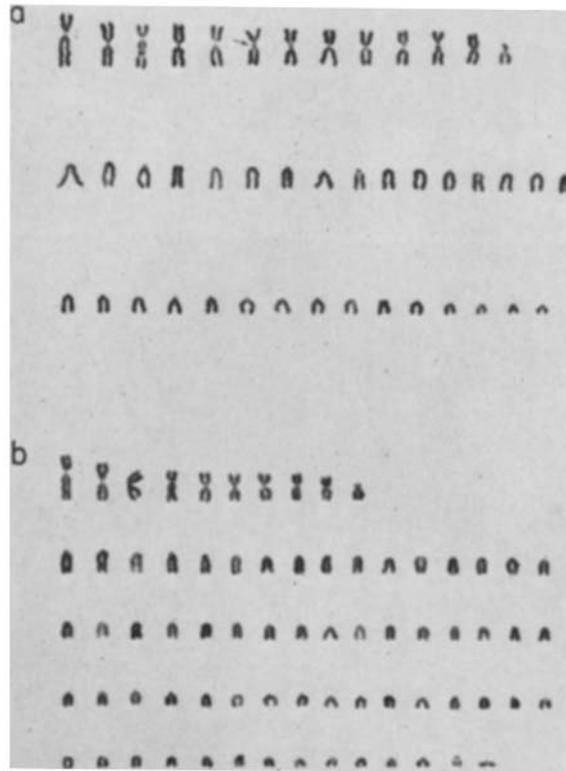


FIG. 3. Karyograms. (a) LMTK⁻. 44 chromosomes, including 13 metacentrics. Note chromosome with double centromere (arrow), possibly because of fusion of two acrocentrics. $\times 1350$. (b) Hybrid LD₇. 72 chromosomes, including 10 metacentrics. $\times 1250$.

TABLE II
Chromosomes of Macrophage-L Cell Hybrids

Cell type	Range	Mean	Metacentrics
LMTK ⁻	42-50	46	11-13
LD ₃	79-96	88	10-13
LD ₄	71-91	81	10-13
LD ₅	75-95	86	10-13
LD ₇	63-80	73	10-13

* 25 metaphases counted/cell line.

absence of antibody, but such binding was suppressed by using antibody-coated SRBC.

Experiments were done to exclude masking of the receptor similar to that found in macrophage-melanoma cell heterokaryons (13). Hybrids were treated with different doses of trypsin, in the range 0.1–250 $\mu\text{g}/\text{ml}$, to ensure that a hypothetical hybrid receptor was not trypsin sensitive and therefore destroyed during “unmasking.” No receptor was demonstrated after trypsinization.

TABLE III
*H-2 Antigens of Macrophage-L Cell Hybrids**

Cell type	Antiserum	Dilution of antiserum used						
		0	1/10,000	1/3000	1/1000	1/300	1/100	1/40
L	Anti-k‡	—§	1+	4+	4+	4+	2+	—
	Anti-d	—	—	—	—	—	—	—
LD ₃	Anti-k	—	4+	4+	4+	4+	2+	N.E.
	Anti-d	—	—	4+	3+	—	—	—
LD ₄	Anti-k	—	2+	3+	4+	3+	N.E.	N.E.
	Anti-d	—	1+	3+	4+	3+	N.E.	N.E.
LD ₅	Anti-k	—	2+	3+	4+	3+	2+	N.E.
	Anti-d	—	—	3+	3+	2+	—	N.E.
LD ₇	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 1/1000).

‡ Anti-k = (C57BL/6 × BALB) F₁, anti-BP8(C₃H) = bd anti-k. Pool EE. Lymph node titer 1/5000. Anti-d = C₃H/An anti-meth A(BALB/c) = k anti-d. Pool EI. Lymph node titer 1/2000.

§ — = negative.

|| N.E. = not examined.

TABLE IV
ATPase Activity of Macrophage-L Cell Hybrids

Cell type	$\mu\text{M P}_i/\mu\text{g protein for 30 min} \times 10^{-3}$	Macrophage activity
DBA/2 macrophages	491 \pm 36	100
LMTK ⁻	0 \pm 0; 9.6 \pm 2.0*	0; 2
LD ₃	51 \pm 3.5; 114 \pm 15	10; 23
LD ₄	77 \pm 1.5; 115 \pm 10	16; 23
LD ₅	62 \pm 2.6	13
LD ₇	60 \pm 6.0; 64 \pm 5.7	12; 13

* Two separate experiments, each in triplicate.

(d) *Complement receptor*: Control macrophages showed 4+ rosette formation when active complement was present on the indicator red cells. L cells were negative, as were the controls with heat-inactivated complement. Macrophages maintained their complement-binding activity during 4 days' cultivation in vitro. Trypsin treatment (10–100 $\mu\text{g}/\text{ml}$ at 37°C for 30 min) abolished rosette formation and treated cells regained their reactivity 1–2 days after cultivation in 199 + 20% newborn calf serum.

The hybrids, however, showed no activity before or after trypsin treatment. Similar studies to those searching for the Fc receptor were again completely negative. The properties of parental cells and hybrids are summarized in Table V.

TABLE V
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 (per cent)	100	0–2	10–23
Fc receptor	+	—	—
Complement receptor	+	—	—

DISCUSSION

The cells produced in the present study are undoubtedly hybrid on the basis of their karyotype, PGI isozymes, and H-2 antigens. We also feel confident that they are true macrophage hybrids; precautions were taken to prevent fibroblast contamination of macrophage cultures; fibroblast-L cell heterokaryons do not form or grow preferentially and several independent clones were isolated.

The hybrids retained 85–100% of the sum of two parent cells' chromosomes, as expected for mouse \times mouse hybrids (7). In certain types of hybrids, chromosomes may be lost preferentially from the genome of the parent with the longer generation time (11). The macrophage-L cell hybrids could therefore have lost mainly macrophage chromosomes. On the other hand, L cells in stationary phase were chosen for hybridization; the macrophage and L cell nuclei of the resulting 1:1 heterokaryons should have entered S in synchrony, perhaps avoiding preferential losses of macrophage chromosomes during mitosis (14). In any event, chromosome loss was limited and macrophage genes for thymidine

kinase, PGI, H-2, and probably ATPase were all retained. It is also unlikely that the genes for macrophage-specific receptors were lost selectively in several independent hybrids. The absence of these particular receptors is therefore because of a failure in expression.

The present studies confirm that H-2 antigens provide useful and sensitive markers to identify cell hybrids (15–17). As a rule H-2 and species antigens are expressed when hybrids retain their genes, except for Ehrlich ascites tumor cells which suppress surface antigens nonselectively after hybridization (18–20). It is known that a proportion of these Ehrlich cell hybrids retain H-2 genes since the antigen may reappear after further chromosome loss (21). The macrophage hybrids differ from previously studied hybrids in that different membrane markers show selective expression; both sets of H-2 antigen are fully detectable, the specific activity of hybrid cell ATPase is intermediate in level and the unique macrophage Fc and complement receptors undetectable. Intermediate levels of gene product have been observed in several other hybrid systems (22–25). Electrical excitability, another specialized membrane function, is preserved in neuroblastoma \times L cell hybrids (26).

Our failure to detect two macrophage-specific receptors in hybrids does not mean that they are absent. We do not know the lower limit in sensitivity of our assays, nor whether a critical receptor density is required to bind the indicator red cells. Mild proteolytic treatment did not unmask these receptors (13) nor did simple variations in the phase of cell growth or cell density. Fc receptor activity increases markedly as promonocytes mature into macrophages (unpublished observations) and macrophages continue to express receptor activity during long-term culture *in vitro*. The relationship between macrophage proliferation and receptor expression is, however, obscure; it is not known if SV40-transformed macrophage cell lines, for instance, retain Fc receptor activity (27, 28).

Many cell-specific properties are lost during long-term cultivation *in vitro* (29). Moreover, hybrid cells commonly lack cell-specific traits expressed by their parent cells in culture (16, 30–35). The selective absence of membrane receptors in macrophage hybrids could be because of low receptor density, incomplete receptor synthesis (36), or some more obscure repressor mechanism. If macrophage receptors are undetectable in conservative mouse \times mouse hybrids because nonmacrophage chromosomes are also present, will they reappear in reduced mouse macrophage \times human nonmacrophage hybrids as human chromosomes are lost (6)? The further study of this receptor in macrophage hybrid cells may prove to be useful in understanding how cells control the expression of cell-specific properties.

SUMMARY

The plasma membrane of the mouse peritoneal macrophage has specific receptors which enable the cell to bind IgG or complement-coated sheep red

cells and is also rich in a divalent cation-dependent adenosine triphosphatase (ATPase) activity. L cells lack these macrophage membrane markers. The question of macrophage membrane receptor expression was investigated in DBA/2 mouse macrophage \times mouse LMTK⁻ cell hybrids produced with the aid of Sendai virus. Three independent clones and one mass culture were isolated by their ability to grow in hypoxanthine, aminopterin, and thymidine (HAT) selection medium. These hybrids retained 85–100% of the sum of two parent cells' chromosomes and expressed several genes derived from both parents, including glucose phosphate isomerase isozymes and H-2 antigens. The hybrids displayed ATPase activity which was intermediate between that of the macrophage and L cell. The macrophage specific receptors for antibody or complement-coated red cells could not be demonstrated on hybrid cells. The selective absence of these receptors is probably because of a failure in gene expression rather than to loss of genes.

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