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MHC antigen expression on bulk isolated macrophage-microglia from newborn mouse brain: induction of Ia antigen expression by γ-interferon

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Summary

Macrophage-microglia were isolated from primary mixed brain cell cultures of normal newborn mice. They were successfully maintained in vitro for at least 8 weeks. Purity of the cultures was 97–100%, as determined by endocytosis of latex beads, non-specific staining through Fc receptors, EA and EAC rosette formation. These cells were non-specific esterase-positive, but peroxidase-negative. Electron-microscope observations revealed morphological similarities to mature macrophages. Isolated macrophage-microglia seldom incorporated [^{3}H]thymidine in vitro. By means of ^{51}Cr release assay, using monoclonal antibodies against mouse major histocompatibility complex (MHC) antigens and complement, we detected class I MHC (H-2) antigen on unstimulated macrophage-microglia, and both class I and class II (Ia) antigens on γ-interferon-treated cells. These observations suggest possible immunoregulatory functions of macrophage-microglia in the central nervous system, as is characteristic of other cells of monocyte lineage.

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Introduction

Lymphokines, especially γ-interferon (IFN), induce class I major histocompatibility complex (MHC) antigens on the surface of most neural cells (Wong et al. 1984; Suzumura and Silberberg 1985, 1986; Suzumura et al. 1986a), and class II MHC antigens on some astrocytes (Hirsch et al. 1983; Fontana et al. 1984; Wong et al. 1984). Recently, we have shown that a neurotropic coronavirus (MHV-A59) infection of the central nervous system (CNS) induces class I antigen, but not class II antigen, on mouse oligodendrocytes and astrocytes (Suzumura et al. 1986b). Another strain of murine neurotropic coronavirus (MHV-JHM) reportedly induces class II antigen expression of 10–20% of rat astrocytes (Massa et al. 1986). Inductions of MHC antigen expression on neural cells, which do not normally express detectable MHC antigens on their surface, suggest the possibility that the CNS cells can interact with or be a target of immunocytes in certain pathological conditions.

Recent studies suggest possible immunoregulatory functions of astrocytes (Fontana et al. 1984, 1986; Fierz et al. 1985; Traugott et al. 1985; Massa et al. 1986). Macrophage-microglia represent another type of cell in the CNS that may have immunoregulatory functions. The origin of macrophage-microglia in the CNS is still controversial. However, evidence suggests their origin from the monocyte (Imamoto et al. 1973; Ling 1981; Polak et al. 1982). Most cells of monocyte lineage, such as Kupffer cells, Langerhans' cells and the alveolar macrophage, express class II antigens and play a key role in initiation or regulation of local immunological events, functioning as antigen-presenting cells or accessory cells (reviewed in Shevach 1985).

We therefore decided to determine whether macrophage-microglia in the CNS have the same immunoregulatory functions as other cells of monocyte lineage. In order to approach this, we isolated a pure population of macrophage-microglia from primary dissociated mixed brain cell cultures of normal newborn mice.

Macrophage-microglia have quite similar, if not the same, properties as the cells of monocyte lineage. By means of ^{51}Cr release assay, we detected MHC class I (H-2) antigens, and both class I and class II (Ia) antigens after γ-IFN treatment, on isolated macrophage-microglia. The results suggest that these cells may participate in immunoregulatory functions in the CNS.

Materials and methods

Animals

AKR/J strain (Jackson Lab., ME) and BALB/c strain (Charles River Labs., MA) mice were used.

Reagents

Monoclonal antibodies against mouse class I MHC antigens; anti-H-$2D^k$,K^k (supernatant of cell line 3-83P), anti-H-$2D^d$,K^d (supernatant, 34-1-2S), and antibod-

ies against mouse class II MHC antigens; anti-I-A^k (immunoglobulin fraction, 26-7-11S), anti-I-E/C^k (ascites, 14-4-4S), and anti-I-A^d (supernatant, 34-5-3S) were obtained commercially (Bionetics Labs., SC).

Isolation of macrophage-microglia

Primary dissociated brain cell cultures were prepared in 75 cm^2 culture flasks, and were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS, HyClone Labs., UT), 5 μg/ml insulin, 2 mg/ml glucose, 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin as detailed previously (Suzumura et al. 1984). Small, round and phase-bright cells which appeared to be oligodendrocyte/precursor cells (Suzumura et al. 1984) began to grow on the top layer at 5–7 days in vitro (DIV) (Fig. 1A). They were then shaken off at 10 DIV to obtain oligodendrocyte-enriched cultures. The flasks containing undetached flat cell layers (Fig. 1B) were re-fed with culture medium and cultured in the same manner. One or 2 days after first shaking, irregular phase-bright cells, which were a little larger than oligodendrocyte/precursor cells, appeared on the top layer. These cells became apparent over the entire surface (Fig. 1C) in 2–4 days. Cultures were then shaken on an orbital shaker as for isolation of oligodendrocytes, but at 150–200 rpm rather than 300 rpm. Supernatant media containing detached cells were filtered with 20 μm nylon mesh and were placed in an uncoated Petri dish. They were incubated in 5% CO_2-95% air atmosphere at 37 °C for 5 min to allow cells to attach to a dish. Medium containing unattached cells was removed and each dish was washed twice with culture medium to further eliminate unattached cells. The adherent cells were collected with a rubber policeman, and were plated on a poly-L-lysine-coated coverslip at a density of 1×10^5/12 mm diameter coverslip. Non-adherent cells were again washed off and re-fed with culture medium 1 h after plating.

Identification of macrophage-microglia

Phagocytosis of latex beads. The macrophage-microglia monolayer was incubated with latex beads (1.1 μm, Dow, final dilution 1 : 1000) at 37 °C for 30 min. After rinsing 4 times in a beaker containing MEM, they were fixed with acid alcohol and mounted on a glass slide with glycerol (Suzumura et al. 1984).

Fc receptor detection. Cells were labeled with rhodamine-conjugated goat anti-rabbit immunoglobulins (Cappel, 1 : 50 dilution) in the presence of 0.2% sodium azide at 4 °C and examined with fluorescence microscopy as detailed elsewhere (Raff et al. 1979). EA-rosette formation was also examined to detect Fc receptors using anti-sheep erythrocyte antibody (Cappel, 1 : 128)-coated sheep erythrocyte (Ross 1981).

Complement (C_3) receptor detection. EAC-rosette formation was examined on the macrophage-microglia monolayer. EAC was prepared as detailed elsewhere (Ross 1981) by EA and C_5-deficient mouse serum (AKR).

Cytochemical staining. Cytoplasmic non-specific esterase staining was performed using *l*-naphthyl butyrate as a substrate (Li et al. 1973, modified by

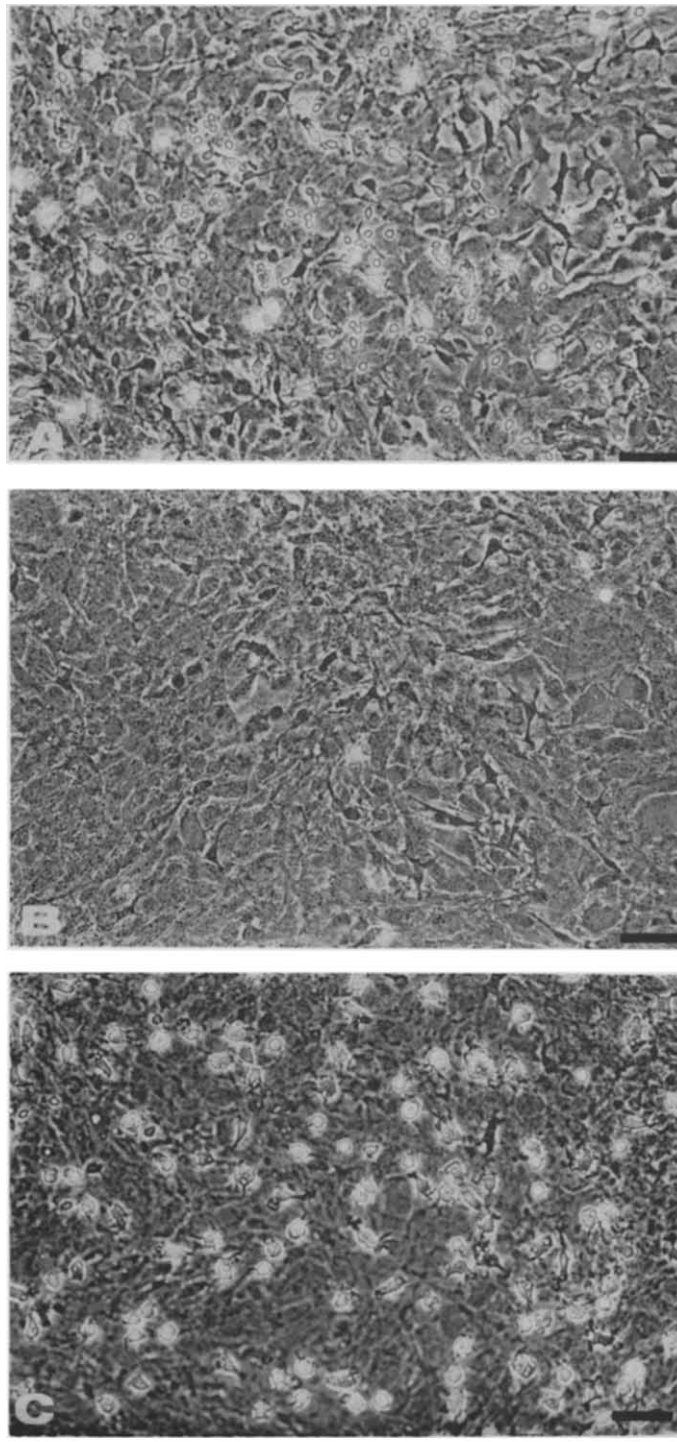


Fig. 1. Primary dissociated brain cell cultures grown in a culture flask. *A*: 10 days in vitro (before shaking). *B*: 11 days in vitro (after shaking). *C*: 15 days in vitro. Bar = 50 μm.

Essayan, in preparation). Peroxidase staining was carried out as described by Kaplow (1981).

Electronmicroscopic observation. Macrophage-microglia cultures of newborn mouse brain were plated on plastic strips (Aclar 33C, 5 mil, Allied Chemical Corp., Morristown, NJ) (Gonatas et al. 1984). After 24 h, the Aclar strips were incubated in fresh medium (Suzumura et al. 1984). Two hours later, the cell cultures were washed 5 times with Earle's balanced salt solution (EBSS), and fixed for 40 min at room temperature (RT) in freshly prepared charcoal-filtered glutaraldehyde (2.5%)/paraformaldehyde (1%) in a 0.1 M sodium cacodylate buffer of pH 7.4 containing 0.025% $CaCl_2$. The plastic strips were then washed 3 times with EBSS and postfixed for 30 min at RT in 1% osmium tetroxide/0.5% potassium ferrocyanide in deionized water. Postfixed cultures were dehydrated by passing through a graded series of alcohol solutions, cleared in propylene oxide, and embedded in Araldite. Thin sections were cut with an LKB Ultramicrotome (LKB Produkter, Bromma, Sweden), stained with uranyl and lead salts according to standard procedures, examined and photographed with a JEOL 100-CX electron microscope using an objective aperture of 40 μm diameter at an accelerating voltage of 80 kV (Gonatas et al. 1982).

[^{3}H]thymidine autoradiography

Proliferation of isolated macrophage-microglia in vitro was examined by [^{3}H]thymidine autoradiography combined with detection of non-specific staining of Fc receptors (Raff et al. 1979). Macrophage-microglia monolayers were cultured with lipopolysaccharides from *E. coli* 0127 : B12 (LPS : Sigma, 0.4–40 μg/ml) or γ-IFN (0.2–100 U/ml) for 2 days. Unstimulated cultures were employed as a control. In the final 16 h, cultures were treated with 0.05 μCi/ml [^{3}H]thymidine (New England Nuclear, spec. act. 2 Ci/mmol). After rinsing in MEM, they were labeled with rhodamine-conjugated goat anti-rabbit immunoglobulins, and were processed for autoradiography as described (Suzumura and Silberberg 1985).

Detection of MHC antigen expression by ^{51}Cr release assay

Macrophage-microglia were isolated at 14–18 DIV, plated on poly-L-lysine-coated coverslips and were cultured for 2 days with or without γ-IFN (20 U/ml) starting 1 day after isolation. Cultures were then incubated with 30 μCi/ml of ^{51}Cr (New England Nuclear, spec. act. 200–900 Ci/g chromium) for 2 h. Optimal conditions for this assay were determined as described elsewhere (Suzumura et al. 1986c). In contrast to isolated oligodendrocytes (Suzumura et al. 1986c), macrophage-microglia on coverslips incorporated ^{51}Cr very well, and were resistant to high concentrations of ^{51}Cr (10–200 μCi/ml for 1–4 h). Since adequate counts for a cytotoxicity assay (10 516 $\pm$ 613 cpm/coverslip, $n = 10$) could be obtained at the concentration of 30 μCi/ml for 2 h, this protocol was employed for this study. After rinsing in MEM 4 times, each coverslip was placed in a well of a 24-well culture plate (Falcon 3047). 50 μl of either anti-MHC antibody or controls was layered on each coverslip with 10% fresh guinea pig serum as a source of complement. Monoclonal antibodies against mouse H-2; anti-H-2D^k,K^k (1 : 2 dilution), and H-2D^d,K^d (1 : 5 dilution)

and against anti-Ia; anti-I-A^k (1 : 10 dilution), anti-I-E/C^k (1 : 10 dilution) and anti-I-A^d (1 : 1 dilution) were used. Supernatant media of non-producing mouse myeloma cell line SP2/0, to which 10 μg/ml of IgG was added (Cappel) and MEM, were used as controls for the above monoclonal antibodies. The plate was kept in a humidified, 5% CO_2 incubator at 37 °C for 2 h. Then, 250 μl of MEM was added to each well to give a total volume of 300 μl. Supernatant fluid was collected into a small glass test tube using an automatic pipettor (Suzumura et al. 1986c). Thereafter, 300 μl of 1 N NaOH was added to each well to lyse the cells. The cell lysate was also collected in a test tube. Each tube was assessed for radioactivity in a Packard gamma counter. Percent ^{51}Cr release was calculated as follows:

$$\text{Percent } ^{51}\text{Cr release} = \frac{\text{cpm of supernatant}}{\text{cpm of supernatant} + \text{cpm of cell lysate}} \times 100$$

Results

Isolation of macrophage-microglia

The cell yield by this method was $3–8 \times 10^5$/flask. This isolation procedure could be repeated on the same flask every 3–5 days, 3–5 times, with a similar cell yield. The cell yield thereafter decreased gradually. Only a few cells could be collected after 2 months. Most of the isolated adherent cells (97–100%) ingested latex beads (Fig. 2A), and were stained non-specifically with either anti-mouse or anti-rabbit immunoglobulins labeled with fluorescein or rhodamine (Fig. 2B). Almost all these cells formed rosettes with EA and EAC, but not with E (Fig. 3A, B and C). These cells were successfully maintained in vitro, although the cell density decreased gradually 7–10 days after plating. About 5–10% of the cells survived for up to 8 weeks (Fig. 4A and B).

Almost all macrophage-microglia were positive for non-specific esterase staining (Fig. 5), although some of them were stained weakly. We did not see peroxidase-positive cells in these cultures. Peripheral blood macrophages were examined as a control, and were positive for both non-specific esterase and peroxidase after the same culture period as macrophage-microglia.

Examination of cell cultures by electron microscopy showed cells with perikarya measuring 20–30 μm in diameter, occasional membrane ruffles and long processes (Figs. 6 and 7).

Nuclei of mouse brain macrophage-microglia were oval or elongated of 8–12 μm longest diameter; they usually occupied an eccentric position. Most of the chromatin was dispersed (euchromatin); condensed chromatin (heterochromatin) was at the periphery of the nucleus near the nuclear envelope.

The cytoplasm contained short segments of granular endoplasmic reticulum, a well-developed Golgi apparatus-complex, microtubules, mitochondria with closely spaced cristae, and numerous dense bodies (putative lysosomes) (Fig. 8). Several of these dense bodies measuring 1–5 μm in diameter may represent phagosomes of macrophages and microglia in the 'activated' state. Coated vesicles were observed in

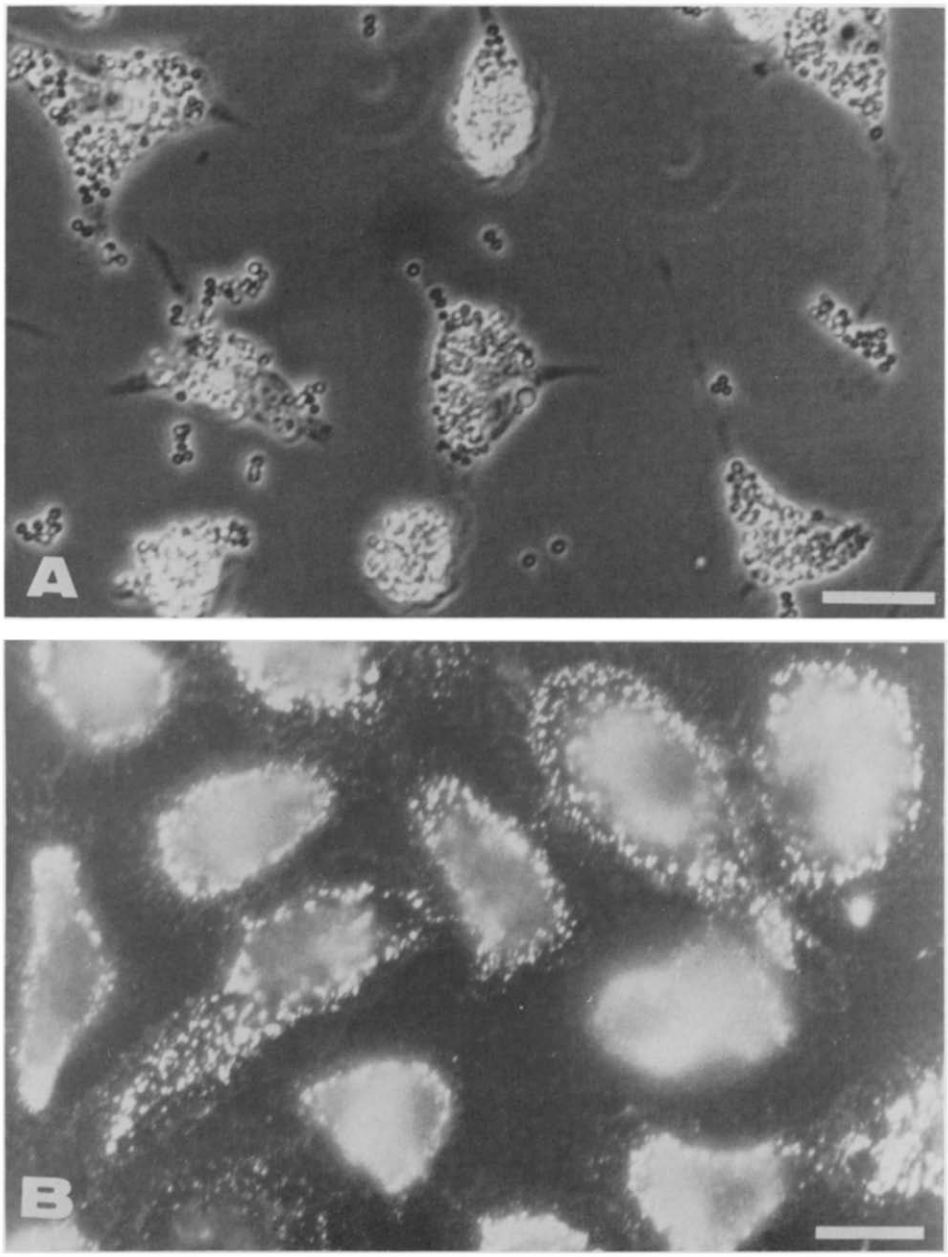


Fig. 2. Isolated macrophage-microglia. *A*: with ingested latex beads. *B*: non-specific staining with anti-rabbit immunoglobulins labeled with fluorescein. Bar = 30 μm.

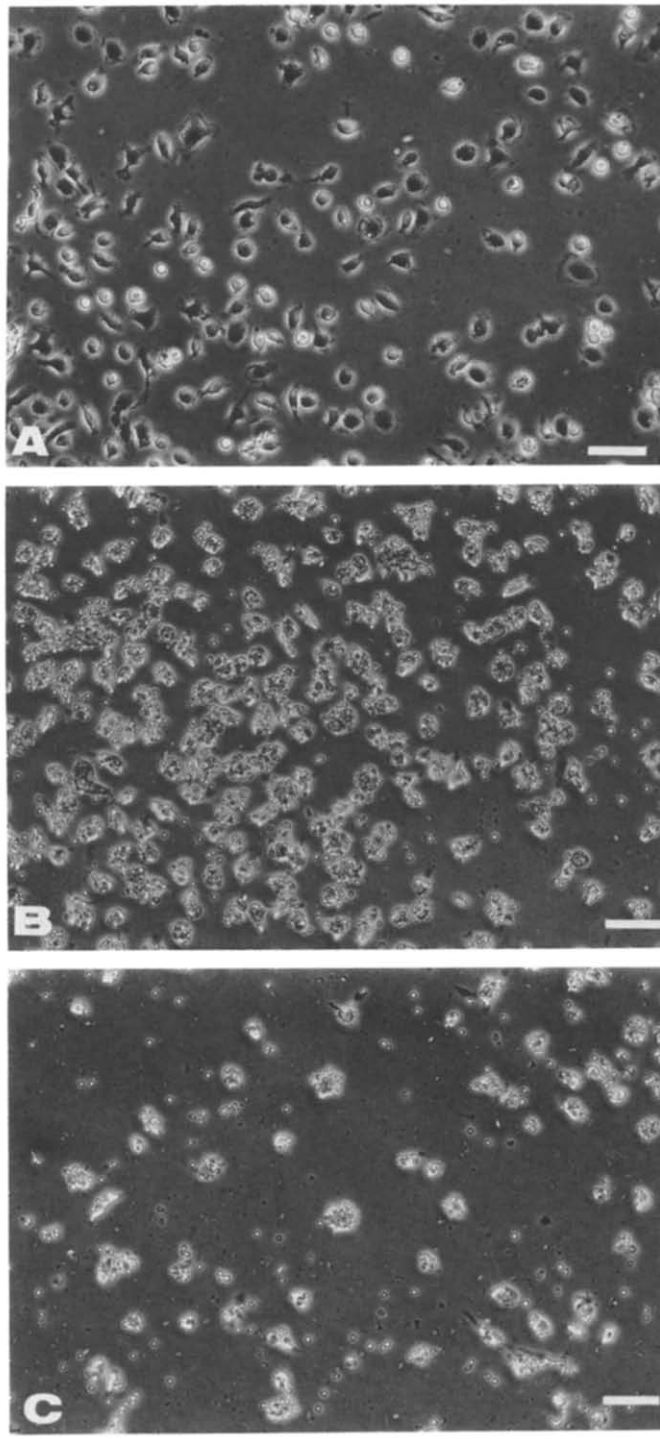


Fig. 3. Isolated macrophage-microglia incubated with E (*A*), EA (*B*), and EAC (*C*). Bar = 50 μm.

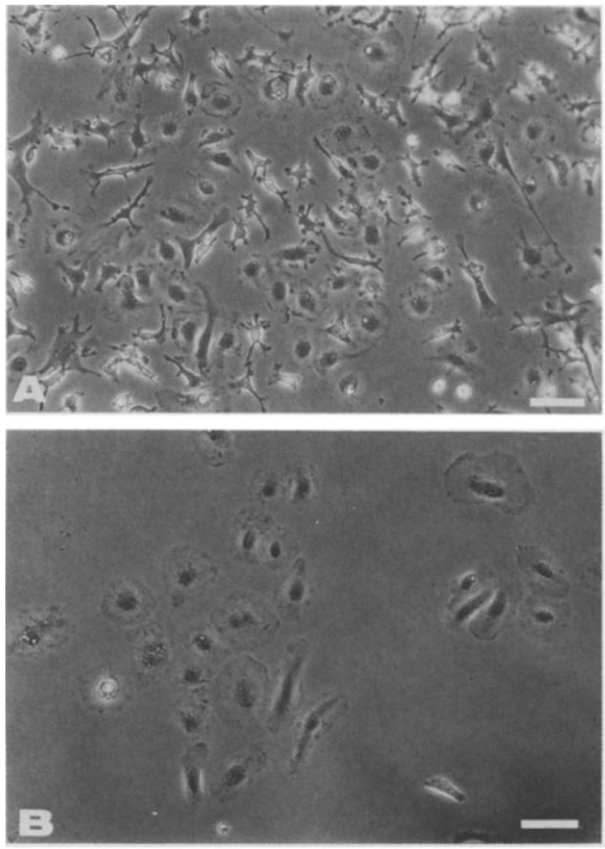


Fig. 4. Isolated macrophage-microglia. *A*: 1 day after isolation. *B*: 8 weeks after isolation. Bar = 50 μm.

TABLE 1

PROLIFERATION OF MACROPHAGE-MICROGLIA

		^{3}H-Labeled cell/Fc-positive cell
Unstimulated		0.2 ± 0.3 (0–0.5)% [a]
LPS	0.4 (μg/ml)	0.1 ± 0.2 (0–0.3)
	4.0	0
	40.0	0
γ-IFN	0.2 (U/ml)	0.2 ± 0.3 (0–0.6)
	2.0	0
	10.0	0.2 ± 0.4 (0–0.8)
	100.0	0.3 ± 0.3 (0–0.6)

[a] Mean $\pm$ standard deviation (and range), $n = 4$.

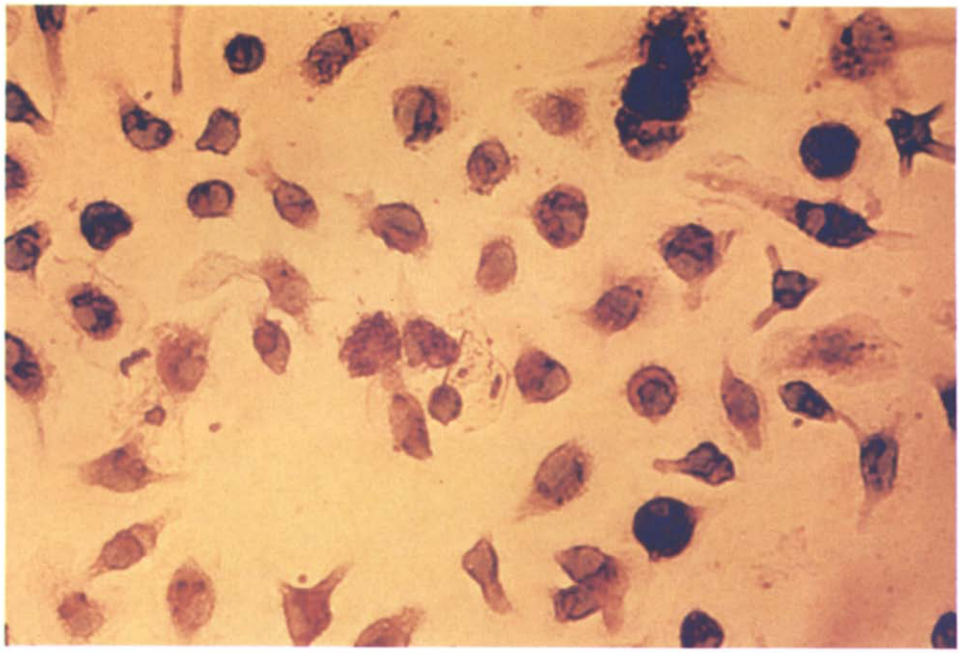


Fig. 5. Non-specific esterase staining of isolated macrophage-microglia.

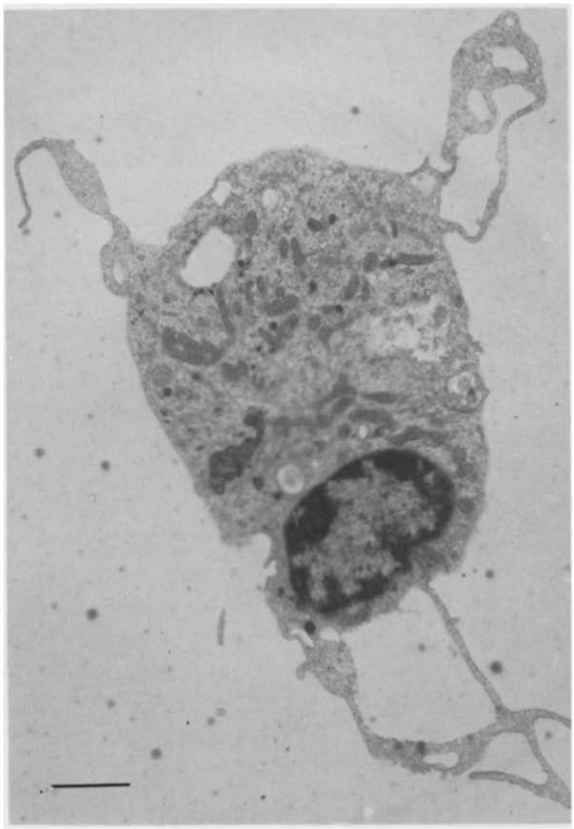
the vicinity of the Golgi apparatus-complex and near the plasma membrane which exhibited several membrane ruffles and coated pits suggestive of ongoing endocytosis. In addition, cells contained membrane-bound vacuoles often enclosing a few vesicles. These vacuoles probably correspond to endosomes of other cells (Gonatas et al. 1984). Several cultured cells possessed long curved cytoplasmic processes filled with actin-like filamentous material similar to microglia in the 'resting' state (Polak et al. 1982).

[^{3}H]Thymidine incorporation

The ratio of [^{3}H]thymidine-positive cells/non-specifically stained cells in unstimulated cultures was 0.2 ± 0.3 (0–0.5)%. Stimulation with LPS or γ-interferon did not enhance [^{3}H]thymidine incorporation (Table 1). Cells isolated at 10 DIV, 17 DIV and 25 DIV of original primary dissociated mixed brain cell cultures gave similar results.

MHC antigen expression

Anti-H-2D^K,K^K, anti-I-A^K and anti-I-E/C^k induced significantly higher ^{51}Cr release in the presence of fresh guinea pig serum from γ-IFN-treated macrophage-microglia of AKR mice (Table 2). In untreated cultures, only anti-H-2D^{k}K^k showed higher ^{51}Cr release. Antibodies against non-corresponding H-2 or Ia showed low background release similar to MEM and SP2/0. This antibody-mediated cytolysis was observed visually as well (Fig. 9A and B). These results indicate AKR



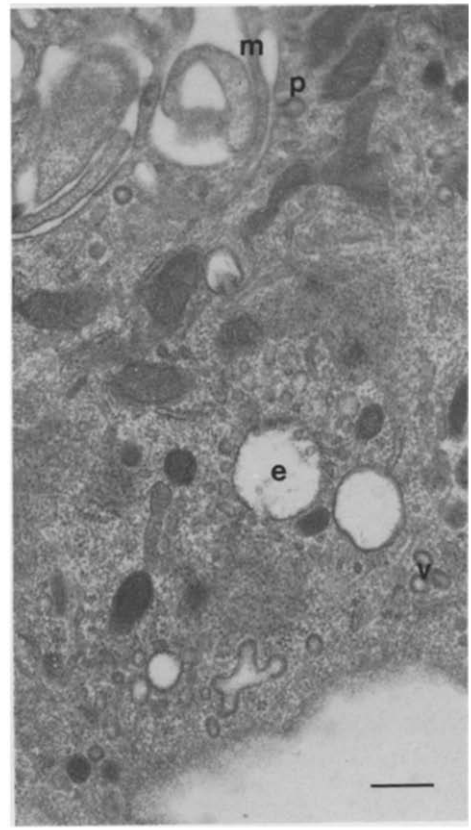


Fig. 6. Newborn mouse brain cultured macrophage-microglia perikaryon and processes. Eccentrically located nucleus shows mostly dispersed chromatin with continuous peripheral condensation; cytoplasmic processes are filled with actin-like filamentous material. Bar = 5 μm.

Fig. 7. The surface of the brain macrophage-microglia cultured cell shows numerous membrane ruffles (m) and coated pits (p). Endosomes (e) and coated vesicles (v) are in the vicinity of the plasma membrane. Bar = 1 μm.

macrophage-microglia express H-2^k antigens, and both H-2^k and Ia^k (I-A^k and I-E/C^k) after γ-IFN treatment. Similar results were obtained in BALB/c macrophage-microglia, except for low ^{51}Cr release with anti-I-A^d. Since anti-I-A^d used in this study requires neat to 1 : 3 dilution to stain lymphoblasts (Suzumura et al. 1986a), these negative results with anti-I-A^d may be related to the low antibody titer of the anti-I-A^d preparation available to us. Alternatively, these results may imply strain differences.

Since some astrocytes treated with γ-IFN reportedly express Ia antigens, potential contamination of the cultures by astrocytes was examined by indirect immunofluorescence using anti-glial fibrillary acidic protein (GFAP) antibody (Suzumura et al. 1984). GFAP-positive astrocytes in these cultures at the time of

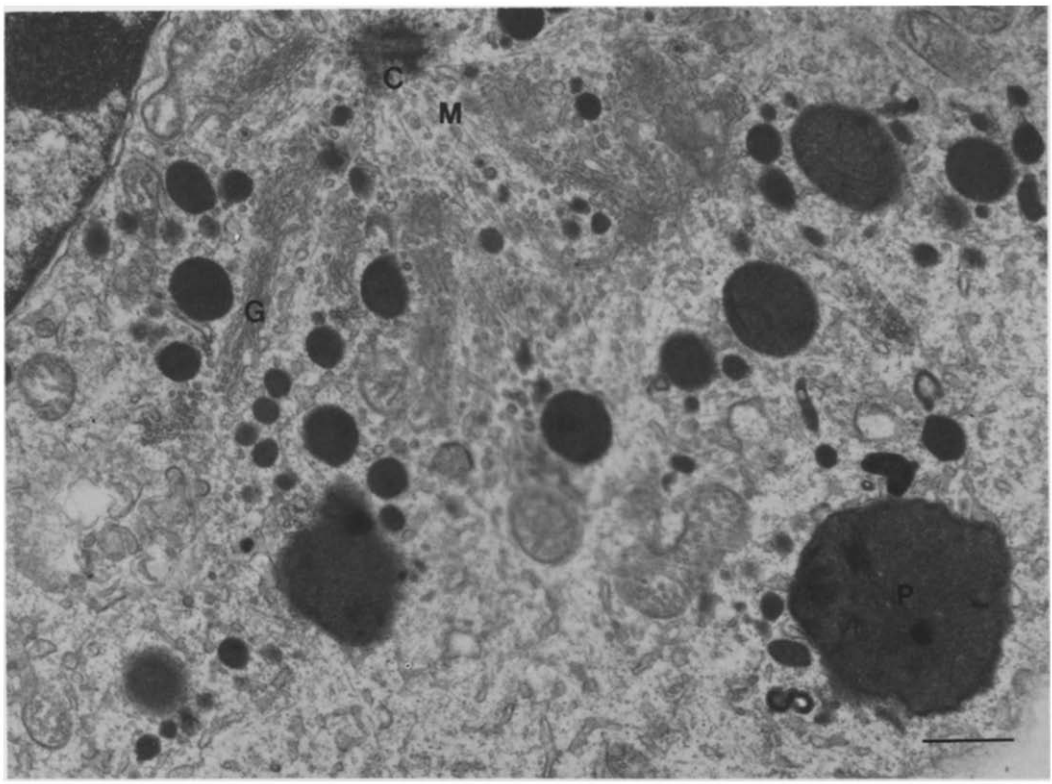


Fig. 8. The cytoplasm of this brain macrophage-microglia cell contains large dense bodies (phagosomes: P), a prominent Golgi apparatus-complex (G) with coated vesicles in its vicinity, microtubules (M), and a centriole (C), Bar = 2 μm.

TABLE 2

MHC ANTIGEN EXPRESSION ON MACROPHAGE-MICROGLIA: ^{51}Cr RELEASE ASSAY

Each value represent mean ± standard deviation of individual percent ^{51}Cr release ($n = 9$), obtained from three separate experiments using triplicate coverslips.

	H-2D^k,K^k	I-A^k	I-E/C^k	MEM	SP2/0	H-2D^d,K^d	I-A^d
4KR							
γ-IFN	47.3 ± 5.0 *	39.5 ± 6.7 *	28.7 ± 9.0 *	15.5 ± 4.2	17.8 ± 3.3	14.5 ± 6.2	16.4 ± 4.2
(−)	31.2 ± 10.9 *	14.9 ± 1.0	15.5 ± 6.1	13.7 ± 2.6	17.3 ± 3.2	14.8 ± 2.2	16.8 ± 4.2
BALB/c							
γ-IFN	14.8 ± 6.8	18.5 ± 4.7	16.9 ± 3.5	13.9 ± 6.1	14.7 ± 2.7	39.4 ± 11.3 *	13.8 ± 7.7
(−)	15.5 ± 3.3	14.2 ± 3.1	13.7 ± 1.7	16.7 ± 1.9	17.9 ± 1.8	35.6 ± 5.4 *	12.9 ± 2.1

* $P < 0.001$ when compared to spontaneous release with MEM.

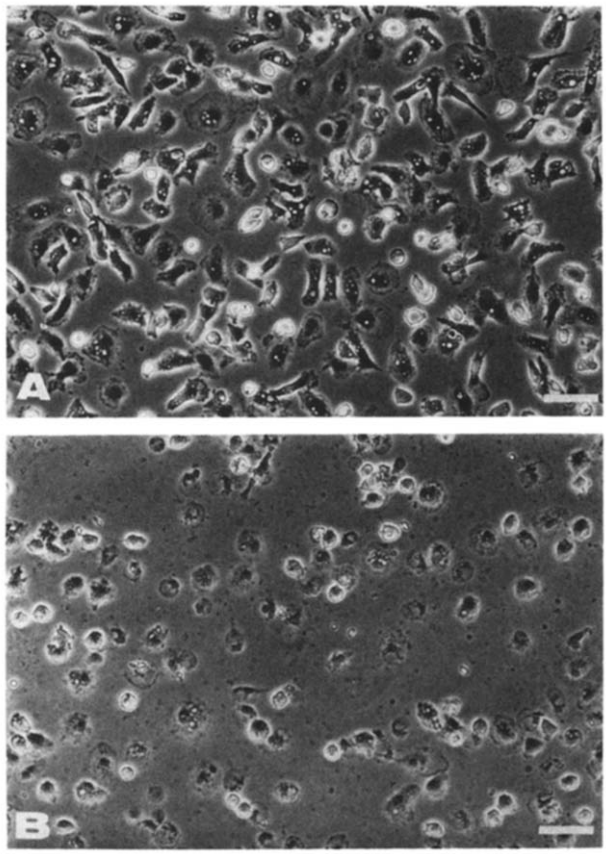


Fig. 9. Macrophage-microglia of AKR strain incubated with SP2/0 (*A*) and anti-I-A^k (*B*) plus 10% fresh guinea pig serum as a source of complement. Bar = 50 μm.

assay, 3 days after isolation, represented less than 1% of the total cells. ^{51}Cr incorporation by astrocyte-enriched cultures was similar to that by macrophage-microglia. Therefore, the results shown above mainly reflect ^{51}Cr release from macrophage-microglia.

Discussion

Giulian et al. (1985) have recently isolated phagocytes from normal rat brain by a similar method, and called them 'ameboid microglia'. However, since neither the origin of microglia nor the relations between macrophages and microglia are clear, we suggest referring to these phagocytic cells in the CNS as macrophage-microglia at this time. Peripheral blood macrophages do not usually survive long in vitro because of their intrinsically short life span and inability to replicate in vitro

(Herscowitz and Cole 1981). By counting red blood cells in initially prepared primary mixed brain cell culture (Dubois et al. 1985a), the roughly estimated contamination of peripheral blood monocyte-macrophages in our study was less than 50/flask and less than 0.5/coverslip. We observed a similar growth pattern of macrophage-microglia in cultures plated on either flasks or glass coverslips. Therefore, it is unlikely that the cells we isolated are blood macrophage contaminated at the preparation of initial cultures.

Isolated macrophage-microglia easily and strongly attached to a plastic Petri dish or glass coverslip. They are non-specifically stained with anti-rabbit immunoglobulins labeled with rhodamine, and phagocytose latex beads. They form rosettes with EA and EAC, but not with E, indicating the presence of Fc and complement receptors. Cytochemical staining revealed non-specific esterase, but not peroxidase, in the cytoplasm. Electron microscopic findings were indistinguishable from those of mature macrophages. We did not find proliferation in our experimental conditions, as determined by [^{3}H]thymidine autoradiography. Dubois et al. (1985a) have also reported that phagocytes in primary dissociated brain cell cultures seldom incorporated [^{3}H]thymidine. These observations indicate that macrophage-microglia in the CNS have very similar properties to macrophages, or the cells of monocyte lineage (Shevach 1985).

We tried to examine MHC antigen expression on macrophage-microglia by means of indirect immunofluorescence. In order to block non-specific binding through Fc receptors, heat-aggregated sera of either mouse, rat, rabbit, guinea pig or bovine were employed. Sodium azide (0.02–10%) or carbonyl cyanide *para*-trifluoromethoxyphenylhydrazone (FCCP 0.2–2.0 mM) was used to inhibit endocytosis of fluorescein-labeled reagents (Gonatas et al. 1977). Sodium azide did not completely inhibit either ingestion of latex beads or endocytosis of fluorescein-labeled reagents. Pretreatment with FCCP (0.2 mM) for 20 min successfully inhibited ingestion of latex beads without obvious morphological changes. However, all the combinations of Fc blocking and inhibition of endocytosis did not completely eliminate non-specific staining with fluorescein- or rhodamine-labeled anti-mouse, rabbit, guinea pig or bovine immunoglobulins or IgG F(ab′).

Prefixation with 1% paraformaldehyde reportedly eliminates non-specific staining through Fc receptors and by endocytosis (Dubois et al. 1985b). However, there was still non-specific binding, when examined by indirect immunofluorescence or radioimmunoassay with SP2/0 containing normal mouse IgG.

Therefore, we examined MHC antigen expression on macrophage-microglia by ^{51}Cr release assay. By this method, we detected H-2 antigens on unstimulated cells and both H-2 and Ia antigens on γ-IFN-treated cells.

Induction of Ia antigen on astrocytes by γ-IFN has been shown by several investigators (Hirsch et al. 1983; Fontana et al. 1984; Wong et al. 1984; Dubois et al. 1985). Fontana et al. (1984) have shown that Ia-positive astrocytes can present antigen to sensitized T cells. However, induction of Ia antigen on astrocytes was observed in a limited age of cultures (Wong et al. 1984) and in a limited population of astrocytes (Hirsch et al. 1983; Wong et al. 1984; Dubois et al. 1985b). On the contrary, lysis or morphological changes of macrophage-microglia by corresponding

anti-Ia antibodies plus complement occurred in most of the γ-IFN-treated cells, which indicate the induction of surface Ia antigen expression on most of these cells.

The present study did not show Ia antigen expression on unstimulated macrophage-microglia. However, since Ia antigen expression on macrophages decreased in long-term cultures, our results do not eliminate the possibility of Ia antigen expression on macrophage-microglia in vivo. The induction of Ia antigen expression by γ-IFN in vitro suggests that impairment of the blood-brain barrier or the presence of activated T cells may induce or enhance Ia antigen expression on these cells. These Ia-positive cells probably play a role in regulation of local immunological events.

The bulk isolation of macrophage-microglia and induction of Ia antigen expression on these cells will be of use in future studies to investigate immunological events in the CNS.

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