Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 7438–7442, August 1991
Immunology

Isolation and direct characterization of resident microglial cells from the normal and inflamed central nervous system

(encephalomyelitis/macrophage/antigen-presenting cell/coronavirus/autoimmunity)

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Communicated by M. Lindauer, May 20, 1991 (received for review January 29, 1991)

ABSTRACT **In addition to the major population of infiltrating leukocytes recovered from inflamed rat central nervous system (CNS), all of which expressed high levels of leukocyte common antigen CD45, many cells were coisolated that were MRC OX42$^+$ (complement receptor 3/CD11b) but expressed low-to-moderate levels of CD45 and major histocompatibility complex (MHC) class I molecules. Most cells from normal CNS, in contrast, lay within this latter, CD45^{low} population. From previous *in situ* immunohistochemical studies, the fortuitously isolated CD45^{low} cells were probably resident (ramified) microglia. Using irradiation chimeras, we show that resident microglia respond to inflammation by upregulating CD45, CD4, and MHC class I molecules with a minority of these cells increasing their expression of MHC class II molecules. A 3- to 4-fold increase in the number of microglia isolated from inflamed CNS provided indirect evidence that the cells had proliferated. In normal CNS, a very small population of blood-derived CD45^{high}-expressing cells are present; most MHC class II expression is associated with these few cells and not with the resident microglia.**

Significant immunological deficiencies in the central nervous system (CNS) include the lack of dedicated lymphatic drainage, low major histocompatibility complex (MHC) expression, and the absence of specialized dendritic antigen-presenting cells (APC, for review, see ref. 1). However, despite this compromised immune status, inflammatory responses are mounted in the CNS against both autoantigens (1) and infectious agents (2). This response indicates not only that the immune system is somehow informed of antigen in this region but also that certain cell types present within the CNS must have the capacity to act as APC and support the development of these responses. The most thoroughly studied candidate APC are endothelial cells of the CNS vasculature (3, 4), astrocytes (3, 5), and microglial cells (6, 7). The latter, in particular, are receiving increased attention as potentially the most important element for development of CNS immune responses (6–10). Moreover, because of surface CD4 expression, this cell type is also considered the most likely focus of human immunodeficiency virus infection in the CNS (11). Microglial cells are now well-defined by *in situ* immunohistochemical staining (7, 12, 13), but the principal markers of this population are also shared by some peripheral blood leukocytes that make it difficult to distinguish between resident microglial cells and those infiltrating from the blood.

In this study we report a method for isolating microglial cells from adult rat CNS. The phenotypic characteristics of isolated microglia defined here by cytofluorographic analysis clearly distinguish them from infiltrating leukocytes. The study, moreover, shows that even normal CNS contains minor populations of leukocytes phenotypically similar to resident microglia but, nevertheless, distinct from them.

MATERIALS AND METHODS

Animals and Irradiation Chimeras. Three- to four-week- and 8-week-old female Lewis (LEW, *RT1^l*) and 8-week-old Brown Norway (BN, *RT1^n*) and (LEW × BN)F$_1$ rats were obtained from Zentralinstitut für Versuchstierzucht (Hannover, F.R.G.). All animals were specific pathogen free. Chimeras were prepared by irradiating young LEW rats with 1000 rads (1 rad = 0.01 Gy) of γ (^{137}Cs) and immediately injecting them i.v. with 5×10^7 viable (LEW × BN)F$_1$ bone marrow cells.

Monoclonal Antibodies (mAbs), Cell Labeling, and Cytofluorographic Analyses. *Rat monoclonal strain-specific alloantibodies.* mAb R2/15S (rat IgG2a) that binds to a determinant on the polymorphic classical MHC class I molecule (*RT1.A*) and is positive on LEW (*RT1.A^l*), but not on BN (*RT1.A^n*), was purified from tissue culture supernatants by anti-rat IgG affinity chromatography. mAb YR5/12 (rat IgG2b), which has reciprocal reactivity on these strains (*RT1.A^{l-}*, *RT1.A^{n+}*) was purified by cation-exchange chromatography from ascitic fluid. Both mAbs were biotinylated for use. Another rat IgG2b mAb (JYI/232) was used for blocking purposes (see below). All three mAbs are listed in ref. 14.

Mouse mAbs. Tissue culture supernatants were used in all studies. Supernatants were generated from hybridomas provided by A. Williams (Medical Research Council Cellular Immunology Unit, Oxford) and Thomas Hünig (Würzburg, F.R.G.). mAbs were as follows: MRC OX1 (anti-rat leukocyte common antigen CD45, ref. 15), MRC OX6 [anti-rat monomorphic MHC class II, RT1B (I-A)], MRC OX8 (anti-rat CD8), MRC OX12 (anti-rat κ chain), MRC OX21 (anti-human C3bi and not rat cells), MRC OX26 (anti-rat CD71, transferrin receptor), MRC OX42 (anti-rat CR$_3$/CD11b, ref. 16), W3/25 (anti-rat CD4), and R73 [anti-rat $\alpha\beta$ T-cell receptor (TCR)]. (See refs. 17–19 for cross-reference details of mAbs.)

Cells were double-labeled with mouse and rat mAbs by resuspending in undiluted mouse mAb and biotinylated R2/15S or YR5/12 mAb to give a final concentration of 15–20 μg/ml of the latter two mAbs. A 1:50 final dilution of nonbiotinylated JY1/232 mAb ascites was included when cells were labeled with YR5/12 mAb to block nonspecific

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Abbreviations: CNS, central nervous system; MHC, major histocompatibility complex; APC, antigen-presenting cells; LEW, Lewis rat; BN, Brown Norway rat; mAb, monoclonal antibody; TCR, T-cell receptor.

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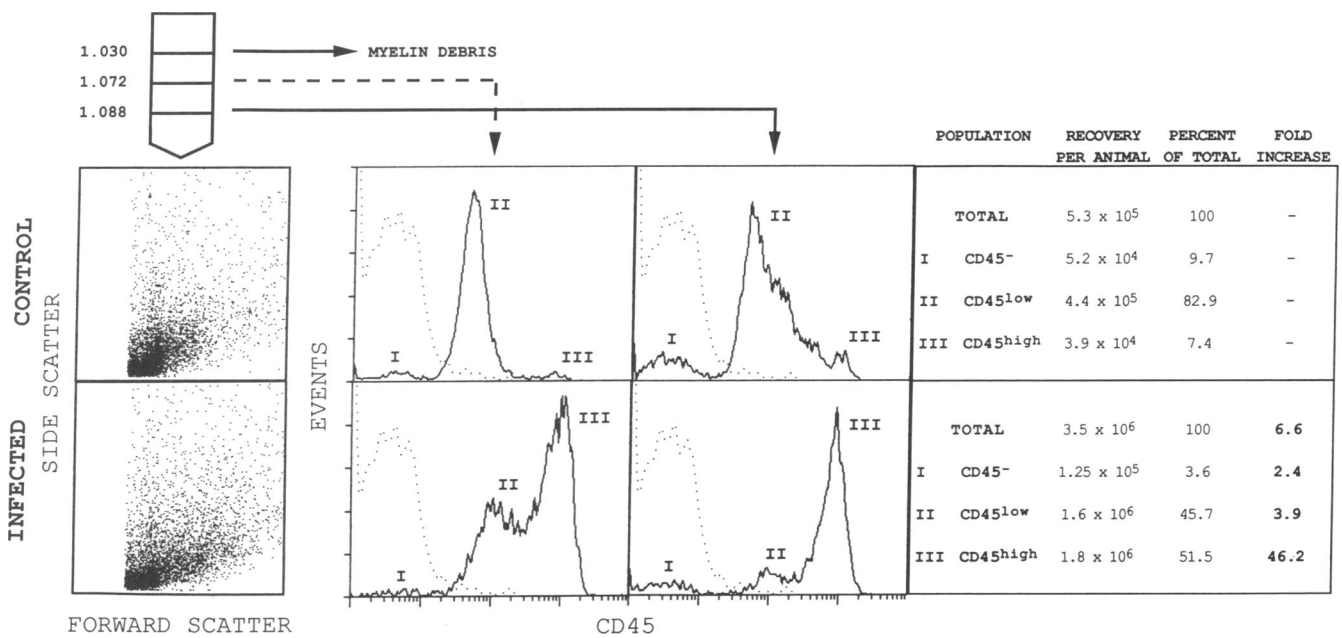
binding of YR5/12 mAb to cells via IgG2b Fc–Fc receptor interaction. mAbs were incubated for 1 hr at 4°C and washed; then a mixture of rat immunoglobulin-absorbed fluorescein isothiocyanate–donkey anti-mouse immunoglobulin at 10 μg/ml (Dianova Hamburg, F.R.G.) and streptavidin–phycoerythrin at 20 μg/ml (Serotec) were added and incubated for 1 hr at 4°C. Cells were washed, and 5–10 × 10^3 live gated events were assessed on a FACScan (Becton Dickinson) using Consort 30 analysis software.

Isolation of Microglia and CNS-Associated Leukocytes. Animals were killed by an ether overdose and perfused via the carotid artery with 200 ml of cold phosphate-buffered saline at an approximate hydrostatic pressure of 1 m of water. The brain and spinal cord were removed, transferred to ice-cold Hanks' buffer/3% fetal calf serum, and minced through a stainless-steel sieve. The dissociated material (both that passing through the sieve as well as that remaining in the sieve) was collected by centrifugation at 170 × *g* for 10 min at 4°C in a 50-ml tube. Each brain/spinal cord was enzymatically digested for 60 min at 37°C with 1.4 ml of 0.75% (wt/vol) type II collagenase (0.95 unit/mg, Serva) and 10^4 units of DNase I (Sigma) in dissociation buffer (42 mM $MgCl_2$/23 mM $CaCl_2$/50 mM KCl/153 mM NaCl). The digested CNS of one animal was pelleted and resuspended in 10 ml of isotonic Percoll (Pharmacia) at 1.098 g/ml diluted in Hanks' buffer, resulting in a density of 1.088 g/ml. This suspension was underlayered with 5 ml of Percoll at 1.122 g/ml and subsequently overlayered with 9 ml each of Percoll at 1.072 g/ml and 1.030 g/ml and 9 ml of Hanks' buffer. The tube was then centrifuged at 1250 × *g* for 45 min at 20°C (acceleration time of 1 min and deceleration time of 5 min). Cells were collected from the 1.072 g/ml and 1.088 g/ml interfaces. Viability was determined by trypan blue exclusion after washing once in Hanks' buffer. Insufficient perfusion of the CNS was indicated by the presence of erythrocytes on top of the 1.122 g/ml interface or in the pellet. In this case the isolated cells were discarded.

Induction of CNS Inflammation. Virus stock in Eagle's minimal essential medium tissue culture medium was derived from the neurotropic wild-type JHM strain of murine hepatitis virus (20) by one passage of the virus through mouse brain followed by one passage over SAC(-) cells. Normal or chimeric rats were intracerebrally inoculated with between 0.5 and 1.0 × 10^3 plaque-forming units of the virus in a volume of 40–80 μl. Control animals either were not injected or were injected with Eagle's minimal essential medium alone. Rats were killed 7 days after infection for isolation of microglia and infiltrating leukocytes. Experimental autoimmune encephalomyelitis was induced in chimeric rats by the subcutaneous injection of myelin basic protein in Freund's complete adjuvant, as described (17).

RESULTS AND DISCUSSION

Coisolation of a CD45^{low} Cell Population with CNS Inflammatory Cells. In normal (nonirradiation chimeric) 3- to 4-week-old animals infected with the neurotropic murine hepatitis virus strain JHM, three distinct populations of cells defined by staining with an anti-rat CD45 mAb were detected: (*i*) a high-expressing population, which was in the majority (CD45^{high}); (*ii*) a low-expressing population (CD45^{low}); and (*iii*) a minority population negative for this marker (Fig. 1). Further studies with control (noninfected, nonirradiation chimeric) animals also revealed these same three populations but with the CD45^{low} cells as the major population (Fig. 1). It has been generally accepted that cells with densities typical of infiltrating leukocytes could not be recovered from normal CNS (for example, see ref. 21), but the gradient technique used here appears to have resolved this problem. Scatter profiles of cells from the two groups of rats were very similar, except for the presence of some cells with high forward scatter (presumably T blasts) in the infected animals (Fig. 1). All three populations in animals with CNS inflammation had increased in number compared with control rats (table of Fig. 1); the relative increase in the order CD45^{high} >> CD45^{low} > CD45$^-$ indicated that the cells constituting these populations had either infiltrated the CNS and/or had proliferated. Note also that fluorescence intensity of the CD45^{low} population clearly increased in animals with an inflammatory infiltrate. Staining of these same populations with the MRC OX42 antibody showed positive staining of most cells recovered from control rats as well as a high percentage of cells from



POPULATION		RECOVERY PER ANIMAL	PERCENT OF TOTAL	FOLD INCREASE
	TOTAL	5.3 x 10^5	100	-
I	CD45$^-$	5.2 x 10^4	9.7	-
II	CD45^{low}	4.4 x 10^5	82.9	-
III	CD45^{high}	3.9 x 10^4	7.4	-
	TOTAL	3.5 x 10^6	100	6.6
I	CD45$^-$	1.25 x 10^5	3.6	2.4
II	CD45^{low}	1.6 x 10^6	45.7	3.9
III	CD45^{high}	1.8 x 10^6	51.5	46.2

FIG. 1. Coisolation of CD45^{low} cells with CNS inflammatory leukocytes. Cells were isolated from the CNS of 3- to 4-week-old control or coronavirus JHM-infected LEW rats. The scatter profile is from pooled interfaces of 1.072 and 1.088 g/ml, as are the recovery figures. The recoveries are each means of three individual animals. The range of recoveries is ±20%. Dotted line, cells from infected rats stained with control mAb MRC OX21. Because of low cell recoveries, MRC OX21 labeling was not always done on cells from control animals.

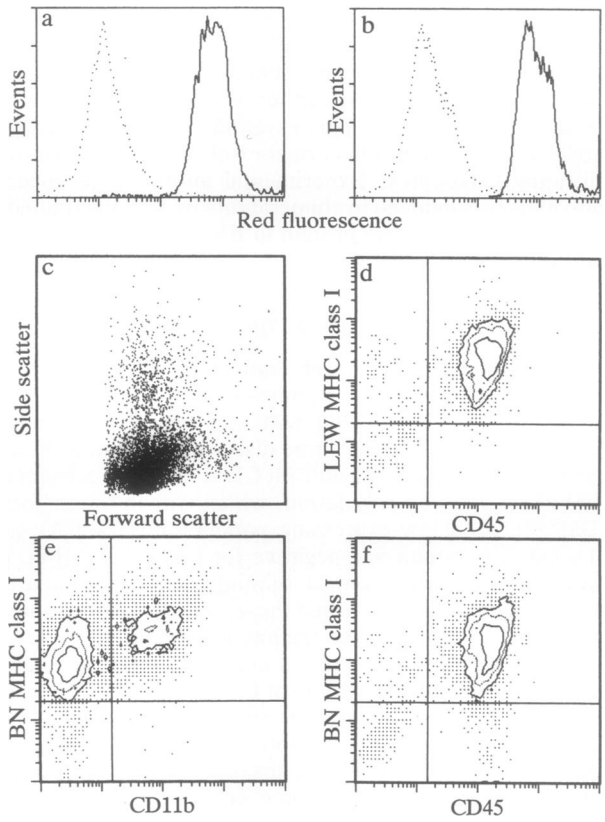


FIG. 2. Complete chimerism for CD11b$^+$ cells. Cervical lymph node cells of adult LEW (*a*) and BN (*b*) rats or peripheral blood leukocytes of (LEW × BN)F_1 → LEW irradiation chimeras 4 weeks after chimera establishment (*c–f*) were stained for cytofluorographic analysis. (*a*) ···, YR5/12 mAb; —, R2/15S mAb. (*b*) ···, R2/15S mAb; —, YR5/12 mAb. (*c*) Peripheral blood leukocytes were prepared by defibrination and hypotonic lysis of erythrocytes, and scatter profiles show distinct lymphocyte, monocyte, and granulocyte populations. (*d–f*) LEW MHC class I (R2/15S) and BN MHC class I (YR5/12).

infected animals. Based on information from *in situ* immunohistochemical analyses (7, 12, 13, 16), we considered that the CD45^{low} population was probably microglia. In normal CNS, histological investigations indicate that microglia are the least frequent neuroglial cell (22) and probably represent <1% of all cells (6). Thus, the total recoveries indicated in Fig. 1 for 3- to 4-week-old rats and, typically, two to three times this number from the adult rat (data not shown) appear reasonable.

In rats with experimental autoimmune encephalomyelitis, comparable populations were recovered. However, the CD45^{high} population was so substantial that the CD45^{low} population generally represented <10% of total cell recovery, and further characterization of the latter population was thus more difficult. For this reason, most studies used animals with viral encephalomyelitis.

Resident (ramified) microglial cells have been reported not to exchange to donor type in semi-allogeneic irradiation chimeras (23). Thus, to study the dynamics and phenotype changes of these cells under inflammatory conditions (LEW × BN)F_1 → LEW chimeras were established.

Irradiation Chimeras. A feature of this and other viral encephalomyelitis models is the reduced susceptibility to infection with age increase (24). Therefore, chimeras were established at a young age (3–4 weeks) and injected with virus 4–5 weeks later when a substantial inflammatory response to the injected virus occurred (see below).

Fig. 2 illustrates that by 4 weeks, chimerism was virtually complete for peripheral blood leukocytes. Fig. 2 *a* and *b* first illustrate the strain specificity of the two MHC class I mAbs used. Most importantly, all blood leukocytes in the chimeras that were CD11b$^+$ (Fig. 2*e*) and CD45$^+$ (Fig. 2*f*) were of donor type (BN MHC class I$^+$). Note that a small population of cells exists that were CD45$^+$ but negative for the donor (BN MHC class I) marker (Fig. 2*f*). These host (LEW) cells (Fig. 2*d*) have been positively identified as CD4$^+$ *or* CD8$^+$ $\alpha\beta$ TCR$^+$ T lymphocytes (data not shown) and remain in chimeric animals for many months.

CD45^{low} Cells Are Resident Microglia and Distinct from "Infiltrating" Blood-Derived Leukocytes. The surface phenotype of cells recovered from the normal CNS is given in Fig. 3 and Table 1. Two distinct populations are revealed, and it is the majority, low-level MHC class I-expressing cells (for example, see Fig. 3*b*) that do not label with mAb specific for BN MHC class I on donor cells (Fig. 3 *h* and *i*). However, most cells within this major population are CD45^{low} and CD11b$^+$. Whether the low level MHC class I expression on the microglial cells reflects existence of this molecule at low level *in vivo* or rapid upregulation during preparation of the cells is unclear.

All resident microglia appear very weakly CD4$^+$; ≈16% express this molecule at a level greater than the set marker (Fig. 3 *d* and *k*). However, included in this summation of cells

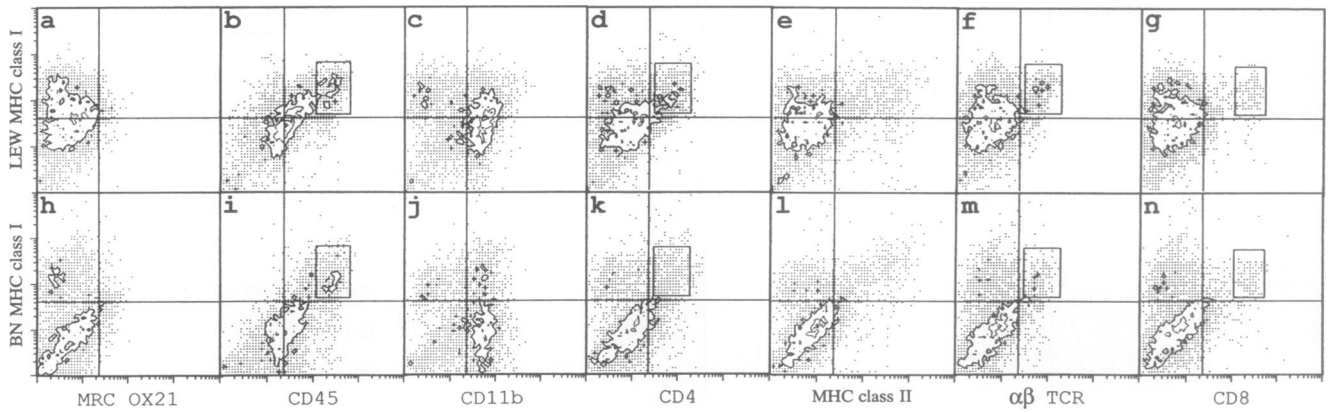


FIG. 3. Resident microglial cells and infiltrating leukocytes in noninfected chimeric rats. Cells were isolated from the CNS of two 8-week-old control (LEW × BN)F_1 → LEW irradiation chimeric rats and pooled. Control animals either were injected intracranially with tissue culture medium only and killed 1 week later or were not injected. The phenotype and number of cells recovered between these two control groups is indistinguishable when isolation is done 7 days after injection. Cells were double-labeled for cytofluorographic analysis. Boxes highlight populations of cells at low frequency of donor type. Some boxed cells in *d* could conceivably be resident microglia. MRC OX21 is a control mouse mAb that does not bind to rat cells.

Table 1. Phenotype of resident and infiltrating CD45$^+$ cells in the normal CNS

Population	% of total*	% positive OX21	CD45	CD11b	αβTCR	CD4	CD8	Surface Ig	MHC class I	MHC class II	TR
A. Resident host	50–70	0	100	82.7	0	16.1	0	0	39.2	6.4	5.6
(microglial cells)		(5.5)†	(29.2)	(57.7)		(13.7)			(39.8)		
B. Infiltrating	15–25	0	100	66.1	33	60.5‡	4.5	<3	100	52	10.2
(donor cells)		(5.5)	(218.2)	(55.1)		(64.1)			(181.8)		

Data represent percent positive cells within population A or B after background subtraction. Data are from the experiment of Fig. 3, in which two animals were pooled, but results are typical. OX21 is a negative control. Percent positivity is derived from the arbitrarily set quadrant markers in Fig. 3. However, all A population cells are weakly MHC class I$^+$ and, probably, very weakly CD4$^+$. TR, transferrin receptor.

*Range recorded in four separate experiments involving three different preparations of chimeric animals. A+B is always <100%. The remaining cells have not been characterized. Population A is defined as those cells that do not express donor class I antigen (defined by mAb YR5/12) and which express low CD45. Population B is composed of those cells that do express donor class I antigen and are high in CD45 expression.

†Fluorescence intensity of 5.5 (OX21) is background, to which other markers can be related. Only intensities for a single marker can be compared between populations A and B.

‡The CD4 molecule is present on CD4$^+$ T cells and macrophages in the rat (25). Thus, ≈28% of infiltrating cells are CD4$^+$ T cells (αβTCR$^+$ minus CD8$^+$).

that are CD4$^+$ but not of donor origin are the CD4$^+$ T cells alluded to above (Fig. 2*f*) that remain of LEW (host) after irradiation and bone marrow reconstitution. A very small proportion (≈6%) of the resident (nondonor) cells (Fig. 3*l*) are also constitutively MHC class II$^+$. Indirect evidence indicates that the microglial cells isolated here are strongly Fc-receptor positive as reported (26) and that the receptor preferentially binds rat IgG of the 2b but not the 2a subclass (data not shown).

Virtually all cells of donor type (those strongly LEW and BN MHC class I positive are highlighted by boxes in Fig. 3) are either αβTCR$^+$ or CD11b$^+$ with few surface immunoglobulin-positive (B cells) detectable (Table 1). This result argues against the possibility that these cells are simply blood contaminants. As ≈ 2/3 of the donor cells in the CNS are CD11b$^+$, one cannot isolate "resident microglia" by this marker. Low CD45 expression is more discriminating (see below). Furthermore, most MHC class II$^+$ cells isolated from the CNS of these control chimeras are donor-derived (Fig. 3*l*), not resident cells, and remarkably, ≈50% of the few donor-type cells isolated are MHC class II$^+$. Some of the CD11b$^+$, CD45^{high} cells of donor origin (a proportion of which may also be MHC class II$^+$) could be the "perivascular" microglial cells reported recently (8, 9). Note that because of the difficulty in removing meninges from spinal cord and cerebellum, the CD45^{high} cells analyzed certainly include a contribution from blood-derived cells present in the meninges. However, analysis of CD45^{high} cells derived from meninges-stripped cerebral lobes indicated that their phenotype was comparable to those described in Fig. 3 and Table 1, including the high level of MHC class II expression, although ≈ 1/3 fewer cells were recovered.

The presence of a small number of constitutively MHC class II$^+$ cells in normal CNS is clearly important in terms of the capacity of T lymphocytes to recognize foreign or autoantigen in a region otherwise relatively deficient in MHC-expressing cells (1).

Of the αβTCR$^+$ cells recovered, both CD4$^+$ and CD8$^+$ are represented at approximately normal ratios (Table 1). The assumed number of αβTCR$^+$ CD4$^+$ T cells (Table 1) may be slightly underestimated, as some of the CD8$^+$ cells could be γδTCR$^+$. The majority of γδTCR$^+$ cells are CD8$^+$ in the rat (27).

The percentage of "donor" cells isolated from these animals (15–25%) is greater than the percentage of putative infiltrating/transient/meningeal-associated (CD45^{high}) cells in younger nonchimeric rats (≈7%) used for Fig. 1. Similar numbers of CD45^{high} cells have also been isolated from adult nonchimeric rats, so this number is not an irradiation artifact but, rather, appears due solely to the age difference. CD45^{high} cells in nonchimeric animals have a similar phenotypic distribution to that shown in Fig. 3 and Table 1 (data not shown).

Upregulation of Multiple Surface Markers on Microglia After CNS Inflammation. Fig. 4 (when compared with its counterpart Fig. 3) and Table 2 clearly illustrate the substantial changes in populations of cells isolated from the CNS 7 days after viral infection. In particular, virtually all cells are now strongly MHC class I$^+$ (compare Fig. 4*a* with Fig. 3*a*)

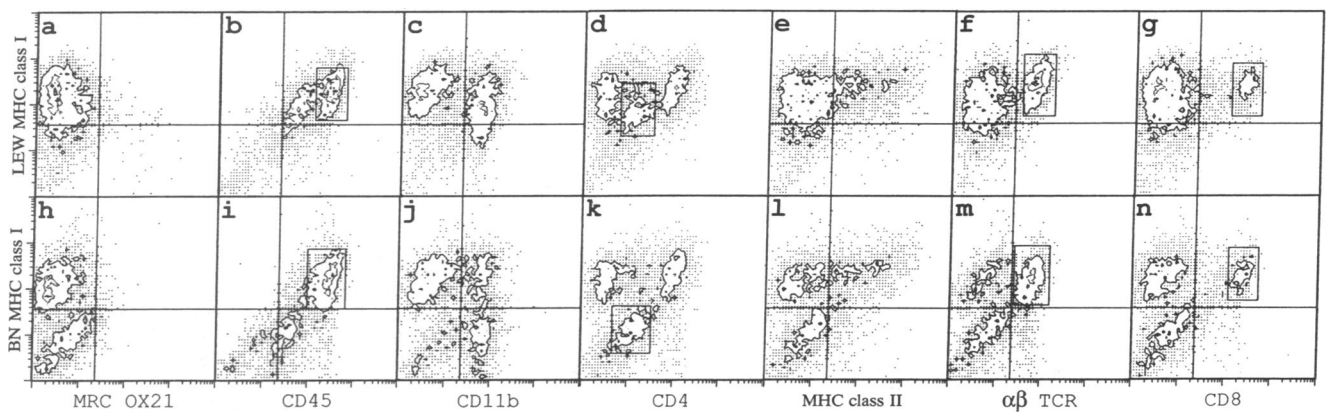


FIG. 4. Resident microglial cells and infiltrating leukocytes in neurotropic virus-infected chimeric rats. Cells were isolated from the CNS of two 8-week-old (LEW × BN)F$_1$ → LEW irradiation chimeric rats injected intracranially 7 days previously with coronavirus JHM and pooled. Boxes highlight populations of donor-type cells, except in *d* and *k*, where resident microglial cells are illustrated. In *b* and *i*, boxed populations represent the same percentage of total cells, but different contour levels are used to accentuate the two populations in *b*. Other details are as for Fig. 3.

Table 2. Upregulation of multiple microglial surface markers after CNS inflammation

Population	% of total*	% positive OX21	CD45	CD11b	αβTCR	CD4	CD8	Surface Ig	MHC class I	MHC class II	TR
A. Resident host	25–45	0	100	85.8	0	22.2	0	0	90.2	23.5	6.5
(microglial cells)		(5.9)	(45)	(76.6)		(16.5)			(88)		
B. Infiltrating	40–50	0	100	32.5	66.9	55.6†	25.9	3.9	100	55.0	5.8
(donor cells)		(4.3)	(260)	(75.9)		(100.9)			(239)		

TR, transferrin receptor.

*Range was recorded as for Table 1. Other data are derived from the experiment of Fig. 4. See Table 1 legend for other details. Data in Tables 1 and 2 were obtained on the same day by using identical cytofluorograph settings and, therefore, are comparable.

†Infiltrating cells are ≈40% $CD4^+$ T cells ($\alpha\beta TCR^+$ minus $CD8^+$).

and over half of the cells are infiltrating donor cells (Fig. 4*h* and boxed populations in Fig. 4 *b*, *f*, *g*, *i*, *m*, and *n*). Upregulation of CD45 (Fig. 4 *b* and *i*) also occurs on the resident microglia (Table 2). As clearly illustrated in Fig. 4*i*, however, no significant overlap occurs at the level of CD45 expression between resident microglia (Fig. 4*i*, BN MHC class I negative) and infiltrating cells (Fig. 4*i*, boxed data), even in inflamed CNS. CD4 expression is upregulated and defines the microglia as a distinct population of cells that are $CD4^{low}$ and express MHC class I of host (Fig. 4*d*, boxed data) but not of donor type (Fig. 4*k*, boxed data); the latter expression is slightly less than that on infiltrating cells.

Around 25% of microglial cells are MHC class II^+, but the major positive population is still the donor, infiltrating cells (Fig. 4*l*), as was found in noninflamed CNS. This result contrasts with the expression of MHC class I, which is found at a relatively high level on virtually all cells. It is noteworthy that the percentage of MHC class II^+ infiltrating cells is greater than the percentage of cells that are $CD11b^+$, indicating that some $\alpha\beta$ TCR^+ cells are MHC class II^+. Activated, but not resting, T cells in rats (28) and humans (29) may express MHC class II molecules. The CD4/CD8 ratio changes significantly from 6:1 in control animals to 1.6:1 in these virus-infected rats, reflecting a selective influx of $CD8^+$ T cells in response to virus. In similar animals with experimental autoimmune encephalomyelitis (data not shown), most infiltrating cells were $CD4^+$ accompanied by a few infiltrating $CD8^+$ T cells, consistent with reports (21).

Are Resident (Ramified) Microglial Cells of Hematopoietic Derivation? Despite their irradiation resistance, the present data provide strong evidence that resident microglia are, at some point, of hematopoietic derivation (12). (*i*) Microglia express a range of markers typically associated with hematopoietic cells, in particular CD45. (*ii*) These cells respond actively to inflammation by upregulating a range of surface antigens (Figs. 3 and 4). (*iii*) Evidence is presented here (although indirect) that microglia ($CD45^{low}$ cells) may proliferate, as indicated by the increased cell recovery from an inflamed CNS (Fig. 1). Therefore, microglial cells, like the recently described BMAC-5^+ macrophage population in rat heart (30), are quite probably a type of fixed tissue macrophage with a low turnover rate (22) and by this criterion, as well as by others, are distinct from interstitial dendritic cells (31).

We extend thanks to Hanne Weinand, Marion Zips, and Barbara Bayer for technical assistance. This work was supported by the Bundesministerium für Forschung und Technologie (Bonn) Grant 01Ki8839.

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