Characteristics of in vitro cytotoxic effects of myelin basic protein-reactive T cell lines on syngeneic oligodendrocytes

Kuniyuki Kawai and Burton Zweiman

*Department of Neurology and the Allergy and Immunology Section, University of Pennsylvania School of Medicine,
Philadelphia, PA 19104, U.S.A.*

Summary

We have previously found that Lewis rat myelin basic protein (MBP)-reactive lymphocytes (Lc) were cytotoxic in vitro to cultured syngeneic oligodendrocytes (oligos). We report here additional studies to characterize this reaction. The effector lymphocytes in the cytotoxic reaction are also encephalitogenic as evidenced by the capacity of other aliquots of these cells to transfer experimental allergic encephalomyelitis (EAE). We confirmed that the presence of both MBP and antigen-presenting cells (APC) are required for this in vitro cytotoxic effect. This reaction (measured by ^{51}Cr release from labeled oligos) is dose-dependent on the effector/target ratio with marked ^{51}Cr release at a 20/1 ratio. Effector/target cell contact is required since: (a) ^{51}Cr release is not significantly increased when effector Lc and oligo are separated by a micropore membrane (28% vs. 24% spontaneous release); (b) no cytotoxic activity is present in the supernatant fluid of a toxic reaction. The adhesion of ^{51}Cr-labeled effector Lc to unlabeled oligo is increased in the presence of both MBP and APC (21 ± 1.0% of cell adhering) as compared with effector Lc + APC (12 ± 2.7%), or effector Lc alone (14 ± 2.8%). Surface expression of class I major histocompatibility complex (MHC) antigens was expressed on the surface of the target oligos during this in vitro cytotoxic reaction. This may explain our previously observed MHC restriction in this reaction. The findings described here may explain some of the in vivo pathogenic events in EAE.

Introduction

T cell-mediated immune mechanisms are believed to play a pathogenic role in experimental allergic encephalomyelitis (EAE) induced by sensitization to myelin basic protein (MBP). Evidence cited to support this concept has included: (1) the prominent mononuclear cell accumulation in the lesional areas within the central nervous system (CNS) (Waksman and Adams, 1962; Raine, 1976); (2) in vitro evidence of lymphocyte immune reactivity to MBP (Ben-Nun et al., 1981); and (3) the capacity to transfer EAE with lines of MBP-reactive T lymphocytes (Paterson, 1960). However, the effector mechanisms involved in mediating EAE

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have not been clearly defined. Such mechanisms might be clarified by a better understanding of the interaction of sensitized lymphocytes and oligodendrocytes, the cells responsible for myelin formation and maintenance in the CNS. Several investigators have shown indirect evidences of in vitro damage of oligodendrocytes. Lyman et al. (1987) found that MBP-reactive lymphocytes induced an increased release from CNS organotypic culture of CNPase (cyclic nucleotide phosphatase), a myelin-specific enzyme. Royta et al. (1985) have reported damage of oligodendrocytes in organotypic cultures induced by lymphoid cells obtained from rodents with EAE.

With the more recent availability of cultured oligodendrocytes (oligos) and cell lines highly enriched for MBP-reactive lymphocytes, it is now feasible to determine whether such immunocompetent cells do affect oligodendrocytes in ways that might not be grossly apparent in the usual visual examination of CNS tissue sections.

To explore the effector mechanisms underlying the clinical EAE manifestations, we have developed an in vitro assay of the cytotoxic effect of MBP-reactive lymphocytes on syngeneic oligos. In initial studies, we found that purified Lewis rat oligos are damaged by MBP-reactive lymphocytes in the presence of antigen-presenting cells (APC) and MBP (Kawai and Zweiman, 1988). We report here on an extension of these studies to learn more about the characteristics and underlying mechanisms of this cytotoxic reaction, especially the contact dependency and the effect of soluble factors.

Materials and methods

Oligodendrocyte culture

Lewis rat oligodendrocytes were isolated by a modification of the methods of Suzumura et al. (1984) as described (Kawai and Zweiman, 1988). Briefly, 17- to 19-day fetal brain dissociated cultures were used for oligos and astrocytes. The primary brain culture was established at $37\,^{\circ}C$ in 5% CO_2/air in a 75 cm^2 plastic culture flask (Falcon 3024) in 10 ml Eagle's minimum essential medium supplemented with 10% fetal calf serum, 2 mM glutamine, 2 mg/ml glucose, 5 μg/ml in-

sulin, 50 units/ml penicillin, and 50 μg/ml streptomycin.

After 9–16 days in vitro, oligos were isolated by shaking in an orbital shaker, then plated on 12 mm diameter poly-L-lysine-coated coverglass at a density of 1×10^5 cells per coverglass. When utilized in cytotoxicity studies 3–4 days later, about 75% (range 65–94%) of the cells on the coverglass were galactocerebroside (Gal-C) positive.

Lymphocyte cultures

Male Lewis rats, 3–4 months old, were immunized with 50 μg of guinea pig MBP and/or tetanus toxoid (TT) (Connaught Laboratories) 1 lipid fluidity unit (lfu)/ml emulsified in complete Freund's adjuvant, containing 50 mg of Mycobacterium tuberculosis H37 RA.

Guinea pig MBP was prepared in our laboratory by a modification of the procedure of Deibler et al. (1972). The purity of MBP was confirmed with a single band seen in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Nine to 10 days later the draining lymph node cells were obtained and cultured in RPMI 1640 medium supplemented with 2% of normal rat serum, 5% of NCTC 109 medium (MA Bioproducts), penicillin 100 U/ml, streptomycin 100 mg/ml, Fungizone 25 ng/ml (Gibco Laboratories, Chagrin Falls, OH, U.S.A.). Guinea pig MBP 30 μg/ml or TT 2 lfu/ml was added to replicate cultures.

After 5–7 days, lymphoblasts were separated at the interface of a discontinuous Percoll gradient (1.050/1.065), then cultured in interleukin-2 (IL-2) containing media. The source of the IL-2 was a supernatant of the cultured MLA-144 cells, as previously described (Kawai and Zweiman, 1988). The line cells were restimulated every 7–14 days with MBP or TT for 3 days. Irradiated normal Lewis rat thymocytes were added as APC. After 2–4 weeks the cultured lymphocytes were restimulated as above and then used as effector cells in the cytotoxicity assay and the adhesion assay described below.

When sufficient numbers of lymphocytes were available in individual MBP-reactive cell lines, used for cytotoxicity studies, other cell aliquots

$(5–10 \times 10^6$ cells) were injected intracardially into naive Lewis rats to assess their capacity to transfer EAE over the next 5 days.

Cytotoxicity assay

(1) Target cells. After determination of optimal labeling conditions in preliminary studies, 1×10^5 oligos or control target cells grown on 12 mm diameter, poly-L-lysine-coated coverglasses were incubated with 20 μCi of ^{51}Cr (New England Nuclear, specific activity 200–900 Ci/g chromium) for 2 h at 37°C, washed 3 times and placed into 16 mm diameter wells containing medium.

(2) Effector cells. MBP- or TT-reactive lymphoblasts from antigen-stimulated cultures were separated by Percoll gradient centrifugation. Freshly isolated normal Lewis rat lymph node cells and TT-reactive lymphoblasts were used as control effector cell populations.

(3) Analysis of cytotoxic activity. Cytotoxicity was assessed by release of ^{51}Cr from the isotope-labeled target cells on replicate coverglasses placed in individual wells of multi-well plates (Linbro).

(a) In the standard assay, to each well was then added 1.0 ml of a suspension of the MBP-reactive lymphoblasts or control effector cell populations either: (i) alone, (ii) with added APC, (iii) with added MBP, or (iv) with both APC and MBP. These cell mixtures were then incubated for 6 h at 37°C in triplicate. A different MBP-reactive line was utilized in each experiment. The supernatant fluid was removed and the cell remaining on the coverglass disrupted by adding 1 N NaOH. The removed supernatant and cell lysate from each well were transferred to separate test tubes for gamma counting (Packard). The percentage of ^{51}Cr release was calculated as:

Percent ^{51}Cr release

$$= \frac{\text{cpm of supernatant}}{\text{cpm of supernatant} + \text{cpm of cell lysate}}$$

$$\times 100$$

The ratio of the number of effector/target cells

was 20:1 for all experiments except those studying the effector cell dose dependency of this reaction in which effector/target ratios of 3:1, 10:1, 20:1, and 30:1 were compared.

(b) In experiments designed to determine the need for contact between effector and target cells, the coverglasses containing the ^{51}Cr-labeled target cells were first placed on the floor of triplicate wells as usual. Then, an insert well with a floor consisting of a Nucleopore membrane (0.4 μm pore size) was inserted into the outer well (Corning Transwell 3413). A 0.5 ml suspension of MBP-reactive lymphoblasts, along with APC and MBP, was added to the inner well. In this step, cells which settled on the Nucleopore membrane floor of the insert well are separated from, but only < 1 mm away from, the target cells on the coverglass in the outer well. After 6 h incubation, the contents of the insert chamber and supernatant fluid of the outer well are aspirated and transferred to a tube for gamma counting; the target cells on the coverglass were lysed and then also transferred to a tube for gamma counting, as above. These effects were compared with isotope release from replicate labeled target cells with which triplicate aliquots of MBP-reactive lymphoblasts plus APC and MBP were incubated concomitantly without any intervening barrier. In previous quality control studies, we found that the presence of the insert chamber itself did not significantly affect the cytotoxic effect of MBP-reactive lymphoblasts added directly to the outer well containing the target cells on the coverglasses.

(c) The possible cytotoxic effect on oligos of a humoral factor released from the MBP-reactive lymphoblasts during an incubation with oligos in the presence of MBP and APC was assessed by obtaining the supernatant fluid after 6 h incubation of known cytotoxic MBP-reactive cells with unlabeled oligos (plus APC and MBP). We also obtained the supernatant fluid from the MBP-reactive lymphocyte cultures just prior to the use of these cultured cells to assess their cytotoxic effect on the target oligos. Aliquots of each of these fluids were stored at $-70°$C and later incubated for 6 h with coverglasses containing freshly ^{51}Cr-labeled oligos. Triplicate labeled oligos or coverglasses were also incubated with MBP-reactive lymphoblasts plus MBP and APC.

Adherence studies

The adherence of MBP-reactive lymphoblasts and other lymphoid cells to the oligo target cells was assessed by a modification of an assay used previously in our laboratory (Zweiman et al., 1982). MBP-reactive lymphoblasts were obtained as described above, incubated with 50 μCi of ^{51}Cr sodium chromate/1×10^7 cells on a rotator at 37°C for 1 h. They were then washed 3 times and resuspended in oligo culture medium. Triplicate 1.0 ml suspensions of these cells were added into 15 mm diameter wells containing poly-L-lysine-coated coverglasses, some of which contained unlabeled oligos, as described above. The cell concentrations employed were the same as used in the cytotoxicity studies. After incubation for 4 h at 37°C, the supernatant fluid was removed and replaced with fresh medium and the plates were agitated for 15 s on a vortex mixer to remove loosely attached cells. The supernatant fluid was discarded and the coverglasses were washed and then transferred to test tubes for gamma counting. An aliquot of the labeled MBP-reactive cells used for the incubation was set aside initially for gamma counting. In a similar way, the adherence of ^{51}Cr-labeled TT-reactive Lewis rat lymphoblasts and ^{51}Cr-labeled Lewis rat node cells to these cultured oligos was also assessed.

Studies of MHC expression

Oligos were examined for the surface expression of major histocompatibility complex (MHC) antigen by indirect immunofluorescence staining before and after co-culture with MBP-reactive lymphocyte replicates inducing a cytotoxic effect. Oligos normally do not express MHC antigens except when stimulated in vitro by agents like γ-interferon. Therefore, the cultured oligos were incubated with recombinant rat γ-interferon (Genzyme, Boston, MA, U.S.A., No. 53010, 2 units/ml) for 48 h, washed and then examined for MHC expression. Oligo specimens were incubated with mouse monoclonal antibodies against rat MHC (OX6, OX3, OX18 and OX27) (Bioproducts for Sciences) and washed, followed by fluorescein-conjugated goat anti-mouse immunoglobulin (Tago) and washed. They were then incubated with rabbit anti-galactocerebroside antibody, kindly provided by Dr. Shama Bhat, Department

of Neurology, University of Pennsylvania School of Medicine (diluted 1:100) followed by rhodamine-conjugated goat anti-rabbit immunoglobulin (Tago). The cells were washed and fixed in acid alcohol at -20°C. Each slide was examined for the frequency of fluorescein (anti-MHC)-positive cells in the rhodamine (anti-Gal-C)-positive population of cells with a typical oligo appearance. In quality control studies, we found no non-specific binding of either irrelevant (anti-human CD4) mouse monoclonal antibody or goat anti-mouse antibody to these oligos.

In selected experiments the capacity of antibodies against rat MHC class I antigens to inhibit the cytotoxic reaction was investigated. We utilized: (1) OX18, a mouse monoclonal antibody (Ab) against monomorphic rat MHC-I antigen (Bioproducts for Science); (2) I-169, a mouse monoclonal Ab against Lewis MHC-I (kindly supplied by Dr. H. Kimura, Department of Pathology, University of Pennsylvania School of Medicine). These were utilized in concentrations which bound strongly to normal Lewis rat node cells. They were added along with the MBP + APC + effector cell combination to the oligos.

Results

General characteristics of the cytotoxic reaction

In a total of 15 experiments, lymphoblasts from MBP-reactive cell lines caused significantly increased ^{51}Cr release from ^{51}Cr-labeled oligo target cells after incubation at 37°C for 6 h (Table 1). As we had found previously in a smaller number of experiments (Kawai and Zweiman, 1988), this cytotoxic effect did not occur in the absence of exogenous MBP (Table 1). In the present study, MBP-reactive lymphoblasts depleted of APC were also not cytotoxic even in the presence of added MBP (Table 1). Thus both MBP and APC appear to be required for this in vitro cytotoxic reaction.

The actual extent of ^{51}Cr release from labeled target cells induced by MBP-reactive lymphoblasts (plus MBP and APC) in individual experiments appeared to be affected by a number of factors: (1) there was a significant correlation between the frequency of oligos (galactocerebroside positive) in the target cell monolayer and the

TABLE 1

CYTOTOXIC EFFECTS OF MBP-REACTIVE T CELLS ON OLIGODENDROCYTES

Each value represents the mean $\pm$ standard deviation of 15 experiments.

^{51}Cr release (%)					Spontaneous release (%)
Effector [a] + APC + MBP	Effector + APC	Effector + MBP	Effector only	Normal lymph node cells + APC + MBP	
65.3 ± 8.5 (n = 15) [b]	26.5 ± 8.3 (n = 11)	32.6 ± 9.4 (n = 7)	28.5 ± 7.7 (n = 9)	20.4 ± 7.1 (n = 8)	23.1 ± 7.5 (n = 15)

[a] MBP-reactive lymphoblasts (see text).
[b] Number of experiments.

extent of ^{51}Cr release ($r = 0.67$; $p < 0.01$, Spearman rank correlation); (2) the cytotoxic effect was dose-dependent on the effect/target cell ratio with a 20 : 1 ratio generally sufficient for a strong cytotoxic effect (Fig. 1); (3) however, there are (yet undetermined) factors leading to variable cytotoxic effects of individual MBP-reactive cell lines. For example, most of such lines obtained during a 6-month period in 1988 induced less ^{51}Cr release from labeled oligos than observed by us with MBP-reactive lines obtained earlier or later.

Relation of in vitro cytotoxic capacity to EAE transfer capacity

The MBP-reactive lymphoblasts that were cytotoxic also exhibited encephalitogenic capacity. In
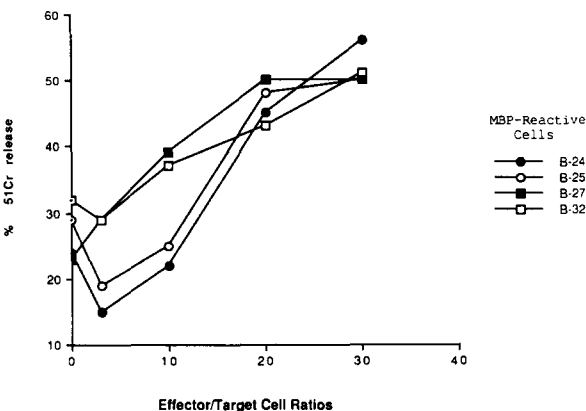

Fig. 1. The effector/target cell dose dependency of the cytotoxic effects of MBP-reactive lymphoblasts on oligodendrocytes. Each line in the figure represents a different population of MBP-reactive lymphoblasts. ^{51}Cr-labeled target oligodendrocytes were incubated with MBP-reactive lymphoblasts plus APC and MBP for 6 h at different effector/target ratios. The first point on the left represents the spontaneous ^{51}Cr release in each experiment.

12 experiments, the numbers of MBP-reactive Lewis lymphoblasts were sufficient to: (1) assess in vitro cytotoxic activity on oligos; (2) inject another cell aliquot (5–10^6 cells) into naive syngeneic recipients, who were then examined daily for clinical signs of EAE. All the MBP line cells that transferred EAE into recipients (signs appearing after a duration of 4–5 days) were also cytotoxic to oligos in vitro and vice versa. We could not compare the intensity of the in vitro cytotoxic effects and the in vivo capacity to transfer EAE because there were not always sufficient numbers of lymphoblasts left over (after that needed for in vitro studies) to inject the same number of cells from each cell line. In contrast, four tetanus-reactive cell line lymphoblasts neither transferred EAE nor were cytotoxic to oligos in vitro. More recently, we have observed occasional MBP-reactive cell lines which were not cytotoxic to oligos. These cells also did not transfer EAE.

Requirement for cell–cell contact between effector and target cells

When the MBP-reactive lymphoblasts plus APC and MBP were added to the insert well of the Transwell apparatus (so that these cells were separated from the labeled oligos in the outer well by a membrane filter (0.4 μm pore size), there were no significant increases in ^{51}Cr release from the target oligos after 6 h incubation (mean = 24% vs. 28% spontaneous release). In comparison, aliquots of the same cytotoxic effector cell combination induced significantly increased (mean = 51%) ^{51}Cr release when incubated directly with the labeled oligos without intervening membrane filters (Table 2).

TABLE 2

EFFECTS OF THE SEPARATION OF THE EFFECTOR CELL POPULATION AND THE TARGET OLIGODENDROCYTES BY A MICROPORE MEMBRANE

Each value represents the mean ± standard deviation.

	^{51}Cr release (%)		Spontaneous release (%)
	Effector [a] + APC + MBP		
	Unseparated well	Separated well	
88-B-45	50.0 ± 0.1	36.7 ± 1.1	35.7 ± 4.3
88-B-44	62.3 ± 3.7	30.8 ± 3.7	35.3 ± 2.2
88-B-49	48.1 ± 1.9	18.7 ± 2.4	18.0 ± 2.2
88-B-50	49.6 ± 2.7	15.5 ± 1.3	19.7 ± 1.0
88-B-51	46.5 ± 4.1	19.9 ± 1.6	31.4 ± 7.4

[a] MBP-reactive lymphoblasts (see text).

Further evidence for the necessity of contact between effector and target cells came from studies of possible cytotoxic activity in the supernatant fluid of these in vitro reactions. Supernatant fluids were obtained after 6 h incubations of the MBP-reactive lymphoblasts (with APC and MBP) and unlabeled oligos (incubation No. 1). These incubations were replicates of similar 6 h incubations of the same effector cell combination with labeled oligos (incubation No. 2). If incubation No. 2 showed significant cytotoxic effects (increased ^{51}Cr release), the supernatant of incubation No. 1 was stored at −70°C and later incubated with fresh ^{51}Cr-labeled oligos for 6 h. No significantly increased ^{51}Cr release was seen as a result of incubation of these supernatant fluids with fresh oligos (Table 3).

TABLE 3

EFFECTS OF SUPERNATANT OF THE CYTOTOXICITY TEST TO ^{51}Cr-LABELED OLIGODENDROCYTES

Each value represents the mean ± standard deviation.

	^{51}Cr release (%)		Spontaneous release (%)
	Supernatant	Effector [a] + APC + MBP	
87-BT-45	37.8 ± 1.3	68.5 ± 3.3	26.4 ± 3.3
87-B-12	34.6 ± 7.4	67.9 ± 2.9	31.4 ± 1.2
87-BT-47	17.7 ± 1.2	65.5 ± 3.2	26.6 ± 2.8
87-B-13	30.1 ± 2.0	67.1 ± 3.2	37.0 ± 2.8

[a] MBP-reactive lymphoblasts (see text).

TABLE 4

ADHESION ASSAY (MBP-REACTIVE LYMPHOBLASTS)

Each value represents the mean ± standard deviation of individual experiments (the mean percentage of adhered cells).

MBP-reactive T cell	Effector [a] + APC + MBP	Effector + APC	Effector only	Control (PLL [b]-coated coverslip)
B-87-1	21.9 ± 2.0	11.8 ± 2.4	15.6 ± 3.8	2.8 ± 1.5
B-87-10	20.3 ± 4.2	8.1 ± 1.0	11.7 ± 3.1	6.8 ± 3.7
B-88-30	20.6 ± 1.3	15.0 ± 0.8	13.8 ± 1.5	9.8 ± 0.7
B-88-32	21.2 ± 3.2	15.9 ± 2.1	17.7 ± 0.8	8.0 ± 1.1
B-88-41	18.9 ± 3.3	12.1 ± 2.2	9.6 ± 1.2	2.1 ± 0.2

[a] MBP-reactive lymphoblasts (see text).
[b] Poly-L-lysine.

To investigate the possibility that humoral cytotoxic factor(s) present during incubation No. 1 had been absorbed out by the target oligos in that reaction (and thus not available to react with fresh oligos), we obtained supernatant fluids from cultures of the MBP-reactive lymphoblasts plus MBP and APC just *before* these effector cells were incubated with the target oligos. These supernates were also not toxic to oligos (data not shown).

Adherence of effector to target cells

A significantly higher percentage of MBP-reactive lymphoblasts adhered to oligos in the pres-

TABLE 5

ADHESION ASSAY (TT-REACTIVE LYMPHOBLASTS AND NORMAL LYMPH NODE CELLS)

Each value represents the mean ± standard deviation of individual experiments (the mean percentage of adhered cells).

| TT-reactive lymphocytes	Lc [a] + APC + TT	Lc + APC	Lc only	Control (PLL [b]-coated coverglass)
TT-16	25.4 ± 5.1	21.2 ± 1.6	21.5 ± 0.1	7.8 ± 0.2
TT-19	16.2 ± 2.9	8.9 ± 1.8	8.0 ± 2.9	1.8 ± 0.7
TT-20	19.4 ± 3.5	11.6 ± 1.4	14.7 ± 2.2	2.1 ± 0.1
Normal LNC [c] (Expt. 1)	2.1 ± 0.1 [d]	2.0 ± 0.1	3.1 ± 0.4	1.8 ± 0.3
Normal LNC (Expt. 2)	3.3 ± 0.3 [d]	3.5 ± 0.6	5.0 ± 0.7	3.2 ± 1.1

[a] Lymphocytes.
[b] Poly-L-lysine.
[c] Lymph node cells.
[d] Normal lymph node cells plus MBP and APC.

ence of APC and MBP (21 ± 0.4%) as compared to 13 ± 0.6% adherence with addition of only APC and 13 ± 1.3% when MBP-reactive lymphoblasts

alone were incubated with the oligos (Table 4). However, this increased adherence of MBP-reactive lymphoblasts was not specific for MBP-reac-
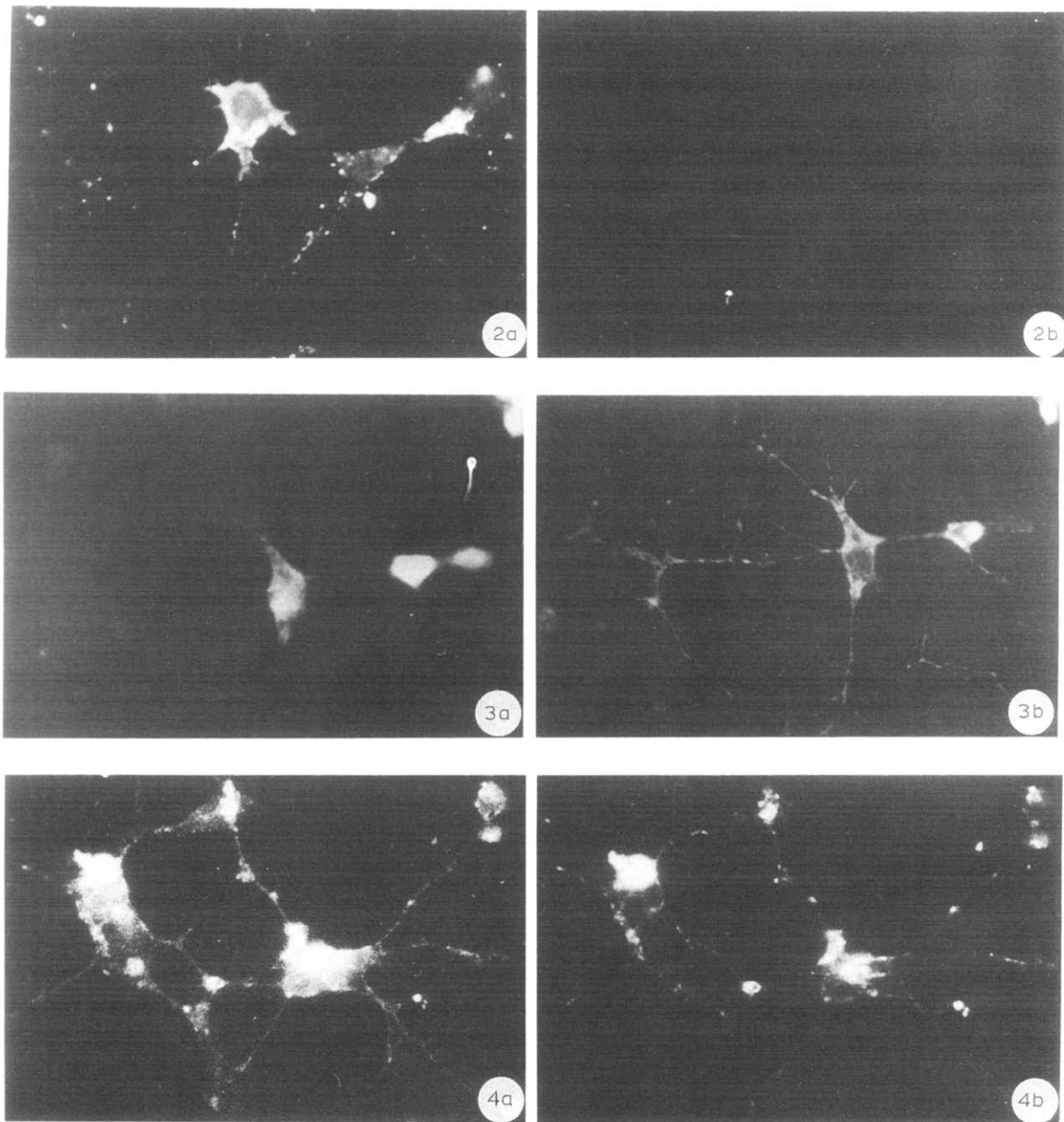
Figs. 2, 3, and 4. Oligodendrocytes simultaneously examined for the surface expression of class I major histocompatibility complex (MHC-I) antigens and galactocerebroside. Fig. 2a and b: untreated cultured oligodendrocytes express galactocerebroside (2a), but express no MHC-I (2b). Fig. 3a and b: γ-interferon-incubated oligodendrocytes express both galactocerebroside (3a) and MHC-I (3b). Fig. 4a and b: oligodendrocytes previously incubated with MBP-reactive lymphoblasts plus MBP and APC for 6 h express both galactocerebroside (4a) and MHC-I (4b).

tive lymphoblasts since about 21% of TT-reactive lymphoblasts were also adherent to oligos when incubated in the presence of TT and APC (Table 5). There was very little adherence of freshly isolated normal lymph node cells to oligos (2.7 ± 1.9%), with no enhancement when these two cell populations were incubated in the presence of MBP and APC (Table 5). These results suggest that any antigen-stimulated lymphoblast might be more adherent than resting node cells to oligos.

Studies of MHC antigen expression

Cultured oligodendrocytes similar to that used as targets in the cytotoxicity assay expressed little or no MHC-I and no MHC-II antigens as determined by binding of specific potent monoclonal antibodies (Fig. 2a and b). Following incubation with γ-interferon, there was a definite surface expression of MHC-I antigen (Fig. 3a and b) but not class II. When cultured oligos were incubated with MBP-reactive lymphoblasts under conditions in which a cytotoxic effect would occur, a search for MHC-I on these oligos was limited by the non-specific fluorescence of dead oligos. However, there was surface expression of MHC-I on oligos still adherent to the coverglass in all of seven experiments (Fig. 4a and b). With OX27 (MHC-I), the staining of the surface MHC-I was weaker than with OX18. There was no detectable MHC-II (OX3 and OX6) expression on the surface of the oligos.

Because of the increased expression of MHC-I antigens on the oligos after incubation with MBP, MBP-reactive lymphoblasts and APC, it was possible that such MHC-I antigens might play a target role in the cytotoxic reaction observed. Therefore we attempted to determine whether addition of the monoclonal anti-MHC-I antibodies employed would inhibit the cytotoxic effects of the MBP-reactive lymphoblasts. These approaches have been limited by a non-specific toxic effect of these monoclonal antibodies on the oligos when used in concentrations sufficient to show strong immunofluorescence when incubated with oligos.

Discussion

As noted earlier in this report, we previously found that MBP-reactive Lewis rat lymphoblasts are cytotoxic in vitro to syngeneic oligodendrocytes (oligos) in a time-dependent reaction (Kawai and Zweiman, 1988). The cytotoxic reaction was dependent on the presence of antigen-presenting cells (APC) and MBP. Histoincompatible Brown Norway (BN) rat oligos were not damaged. However, the cytotoxic effect was not directed simply against any Lewis rat cell since Lewis rat fibroblasts and astrocytes were also not damaged by these MBP-reactive lymphoblasts. The present study addresses some of the unanswered questions about this striking in vitro cytotoxic reaction.

One question deals with the roles of the MBP-reactive lymphoblasts and the APC in the cytotoxic effect. The importance of MBP-reactive lymphoblasts is confirmed by the findings of increasing cytotoxic effects with an increasing ratio of the numbers of the MBP-reactive lymphoblasts to the target oligos. This effector/target dose dependency is seen commonly in other T cell-mediated cytotoxic reactions. The necessity and role of APC in this reaction are less defined. APC may not be required for some cytotoxic effects. For example, Lyman et al. (1986) found that MBP-reactive murine lymphocytes by themselves could damage myelin in syngeneic organotypic brain cultures. The requirement for APC in our in vitro reaction could be due to one or more possible functions of these cells.

(1) The APC could present MBP to the MBP-reactive lymphoblasts leading to further stimulation of the latter cells, with a resultant greater cytotoxic capacity. We do know that not just any antigen-stimulated Lewis rat lymphoblast will be cytotoxic to the oligos since TT-stimulated lymphoblasts from TT-sensitized Lewis rats are not cytotoxic to these oligos (Kawai and Zweiman, 1988). The irradiated APC used in this reaction have no cytotoxic effect of their own on the oligos.

(2) Another possible role of the APC could be to increase the adherence of MBP-reactive lymphoblasts to the target oligos. Indeed, we did find such an increased adherence in the presence of APC. This could be important because a major finding in this study is that the cytotoxic reaction depends on cell–cell contact between the MBP-reactive lymphoblasts and the target oligos. Furthermore, the cell-free supernatant fluid obtained at the end of the 6 h incubation period contained no

humoral factors cytotoxic to fresh oligos. This effector–target cell contact dependency has also been observed in other cytotoxic reactions against cell targets (Shaw et al., 1986; Berrebi et al., 1987). However, increased adherence of lymphoblasts to these cultured oligos is not sufficient by itself for cytotoxic effects. In comparative studies we found that TT-stimulated lymphoblasts adhere as much as the MBP-stimulated lymphoblasts to the oligos. However, only the MBP-reactive lymphoblasts were cytotoxic to these oligos. Thus, the cytotoxic effect on oligos may require adherence of sufficient numbers of lymphoblasts which are immunologically reactive with determinants in the oligos.

That possible mechanism raises a second question addressed in part by these studies: the nature of the oligo target antigens in this immune reaction. As noted above, oligos from histoincompatible BN rats were not damaged by these Lewis MBP-reactive lymphoblasts which were cytotoxic to Lewis rat oligos (Kawai and Zweiman, 1988). This finding raises the possibility that MHC antigenic determinants are part of the target in the cytotoxic reaction. The cytotoxic action of a number of T cell clones against other cell targets appears to be MHC-class I restricted (Forman, 1987). However, unlike the target cells in those models, cultured rat oligodendrocytes do not express either class I or class II antigens, as determined by either immunofluorescence or radioimmunoassay studies. Class I but not class II MHC antigens can be induced on oligos previously exposed to activators like γ-interferon (Wong et al., 1984; Suzumura et al., 1986a, b).

Our findings confirm these earlier reports in that cultured oligos express little or no MHC-I and no MHC-II (MHC-I expression was seen in such oligos previously exposed to γ-interferon). However, after a 6 h incubation of the oligos with the MBP-reactive lymphoblasts + APC + MBP, there was modest expression of MHC-I antigens on many of the oligos in the cytotoxic reaction. The source of the MHC-I antigens seen on these oligos is unclear. It is conceivable that a product released from the lymphoblasts could have stimulated MHC-I expression on the oligos. Indeed, our recent preliminary studies suggest increased MHC-I expression on cultured oligos incubated with the supernatant fluid of the cytotoxic reac-

tion. It is also possible that MHC antigens may have been shed from the lymphoblasts or APC (Hale et al., 1981; Hedlund et al., 1984; Meeusen, 1987). Meeusen (1987) showed that such shed antigen could stimulate a secondary cytotoxic response in the presence of APC. Vass et al. (1984) have speculated that MHC antigens may be bound to altered cells rendering them susceptible to further cytotoxic effects.

However, at least two findings cast some doubt about this postulated role of MHC-I in the target cells. First, the majority of the lymphoblasts in our cytotoxic reaction expressed the putative helper (CD4 equivalent) phenotype. CD4$^+$ cytotoxic cells are thought to be restricted by MHC-II, not MHC-I determinants in their cytotoxic effects (Tite et al., 1985; Nakamura et al., 1986; Sun and Wekerle, 1986).

Second, our preliminary studies do not indicate any increased susceptibility of γ-interferon-treated oligos (expressing MHC-I antigens) to the cytotoxic effect of the MBP-reactive lymphoblasts. Our attempts to determine whether the cytotoxic effects of the MBP-reactive lymphoblasts is inhibited by antibodies against MHC-I determinants have been limited by an inconsistent cytotoxic effect of such antibodies themselves on the target oligos, even when complement was inactivated in all reagents. Although we have not seen impressive MHC-II antigen expression on the target oligos either before or after the 6 h incubation in the cytotoxic reaction, it is possible that a low but sufficient level of MHC-II molecules are expressed. Again, our attempts to inhibit the cytotoxic reaction by OX6 (an anti-MHC-II antibody) have been limited by cytotoxic effects of this antibody on oligos.

It is also conceivable that the resistance of BN rat oligos to the cytotoxic effect of the Lewis MBP-reactive lymphoblasts may be independent of the MHC status of BN rats. Unfortunately, we have not been able to assess the cytotoxic effect of MBP-reactive lymphoblasts from BN rats on BN oligos because it is extremely difficult to induce encephalitogenic lymphoblasts in the EAE-resistant BN strain.

We have not yet identified an oligo-specific component as a target of the cytotoxic reaction observed here. Antibodies against galactocerebro-

side, an oligo surface component, can damage oligos in vitro in the presence of complement, as assessed by ^{51}Cr release (Suzumara et al., 1986c). However, we have found that the presence of similar anti-galactocerebroside antibodies in the absence of complement (which are not toxic) does not inhibit the cytotoxic effects of the MBP-reactive lymphoblasts.

If the in vitro events observed in this study do occur in vivo, one can speculate about the nature of MBP-reactive lymphoblasts passing through the circulation of the central nervous system during actively or passively (cell transfer) induced EAE. Cells such as astrocytes, macrophage-microglia and vascular endothelial cells can express MHC class II antigen (Fontana et al., 1984; Sobel and Colvin, 1985; Traugott et al., 1985; Matsumoto and Fujiwara, 1986) and could conceivably bind and present small amounts of MBP which normally elute from oligodendrocytes and/or myelin within the CNS (Simon and Anzil, 1974; Wisniewski and Lassmann, 1983). If sufficient numbers of activated MBP-reactive lymphoblasts come in contact with such MBP-presenting cells, the MBP-reactive lymphoblasts could be retained at the sites and directly (or indirectly) damage oligodendrocytes. Whether or not such damage is manifested as frank demyelination in the usual pathologic studies may reflect the intensity of the cellular reaction and/or participation by other immunological events.

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