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Protective Effect of Biological Response Modifiers on Murine Cytomegalovirus Infection

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Pretreatment with two biological response modifiers (BRM), OK-432 and PS-K, protected mice from lethal infection by murine cytomegalovirus (MCMV). This was evidenced by an increase in 50% lethal doses and a decrease in titers of infectious viruses replicated in the liver and spleen. Spleen cells from the BRM-treated mice augmented the natural killer (NK) cell activity and suppressed the replication of MCMV in vitro. During MCMV infection, the NK cell activity of the spleen cells was maintained at a high level in the BRM-treated mice, whereas it was severely impaired in untreated mice. The BRM-induced protection was nullified by concomitant administration of antiasialo GM1 antibody. Interferon was neither induced by BRM treatment nor enhanced in BRM-pretreated and MCMV-infected mice. Thus, the protective effect of OK-432 and PS-K seems to be based on activation of NK cells and prevention of MCMV-induced inhibition of the NK cell activity.

A streptcoccal preparation, OK-432, and a protein-bound polysaccharide, PS-K, from *Coriolus versicolor* of the class *Basidiomycetes* have been developed in Japan and widely applied to cancer patients as biological response modifiers (BRM). Although their antitumor effect has been assessed clinically and experimentally (10, 11, 30, 31, 33, 35), it has not been fully determined how they enhance the host defense against tumors. The possible mechanisms reported so far include induction of interferon (IFN) (17, 25) and augmentation of natural killer (NK) cell activity (21, 32–34). Prolongation of the survival of the BRM-treated patients could be attributed partly to increased resistance against opportunistic infections.

It has been shown that NK cell activity correlates with susceptibility to murine cytomegalovirus (MCMV) infection (2, 3, 22, 27). Generally, IFN augments NK cell activity (6, 8, 14), although production of IFN may not be a prerequisite for the development of endogenous NK cell activity against MCMV-infected targets (14). These findings led us to investigate the antiviral effect of OK-432 and PS-K in a mouse-MCMV system. This paper is concerned with nonspecific protection of mice with the BRM against MCMV infection.

MATERIALS AND METHODS

Mice. Female ICR mice were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu, Shizuoka, Japan). They were used for experiments at 5 to 7 weeks of age. Suckling mice were used at 1 to 2 days of age.

Viruses. The Smith strain of MCMV supplied by T. Nakao, Sapporo Medical College, was used throughout the experiments. The attenuated MCMV (CC-MCMV) was obtained after serial passage in mouse embryo fibroblasts (MEF) which were prepared by trypsinization of embryos of the ICR mice and grown in Eagle minimal essential medium (MEM) supplemented with 5% calf serum and 0.12% $NaHCO_3$. The virulent MCMV (SG-MCMV) was obtained by passage in the salivary glands of mice via an intraperitoneal (i.p.) route (19).

Infection of mice. Unless otherwise stated, weanling mice were inoculated i.p. with 10^6 PFU of SG-MCMV in 0.5 ml, and suckling mice were inoculated i.p. with 10^5 PFU of CC-MCMV in 0.1 ml. The 50% lethal dose (LD_{50}) was calculated by the Reed-Muench method.

BRM. OK-432 (Chugai Pharmaceutical Co., Tokyo) was dissolved in sterile physiological saline immediately before use. PS-K (Kureha Chemical Co., Tokyo) was dissolved in sterile physiological saline and sterilized at 100°C for 15 min and stored at −20°C until used. Weanling mice were administered i.p. 0.02 mg of OK-432 or 10 mg of PS-K in 0.5 ml.

Preparation of peritoneal cells and spleen cells. Mice were given BRM on days 3 and 1 before sacrifice. The peritoneal cells were obtained by washing out the peritoneal cavity with 5 ml of Eagle MEM containing heparin and were washed once with Eagle MEM. Spleen cells were prepared from mice that were similarly pretreated. Spleen cells were dissociated with slide glasses from the aseptically removed spleens. After centrifugation at 1,500 rpm for 5 min, erythrocytes were lysed by treatment with 0.83% ammonium chloride for 5 min at room temperature. Spleen cells were washed three times and resuspended in Eagle MEM. Viable cells were determined by trypan blue exclusion.

Replication of MCMV in MEF. Monolayered MEF in a one-side-flattened tissue culture tube (Nunc 1-56758) were infected at a multiplicity of 0.01 PFU of SG-MCMV per cell and cocultivated with the peritoneal cells, the spleen cells, or neither (control). The cultures were incubated at 37°C in 5% CO_2 atmosphere and harvested at various times. After storage at −80°C, viruses were released from infected cells by three cycles of freeze-thawing and assayed by the plaque method.

NK cell activity. Spleen cells prepared as described above were used as effector cells. YAC-1 cells were used as target cells for NK cell activity. These cells were grown in RPMI 1640 supplemented with 10% calf serum. For assay of NK cell activity, YAC-1 cells (10^6 cells in 2 ml) were labeled with 100 μCi of ^{51}Cr for 1 h and then washed three times with RPMI 1640. The labeled YAC-1 cells (10^4) were dispensed into each well of a 96-well microtitration plate (Titertek 76-013-05). Quadruplicated wells were added with spleen cells (10^6 cells per well) at an effector/target ratio of 100:1, and the plate was incubated for 4 h at 37°C. The supernatant fluid in each well was harvested with a supernatant collection system, and the radioactivity was counted in a gamma counter.

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TABLE 1. Effect of BRM on MCMV infection in weanling mice

BRM[a]	Expt no.	No. of mice dead/no. challenged after BRM treatment at day[b]: −5	−3	−2	−1	0	+1	No. of mice dead/no. challenged (control)
OK-432	1	4/4	4/4	2/4	0/4	4/4	3/3	4/4
	2	2/4	2/4	2/4	0/4	3/4	2/4	4/4
	3	ND[c]	ND	ND	0/5	ND	ND	5/5
PS-K	1	2/4	0/4	1/4	2/4	3/4	2/3	4/4
	2	1/4	0/4	0/4	0/4	4/4	2/4	4/4
	3	ND	0/5	ND	ND	ND	ND	5/5

[a] Six-week-old mice were injected i.p. with 0.02 mg of OK-432 per mouse or 10 mg of PS-K per mouse.
[b] Mice were treated with the BRM from 5 days before to 1 day after infection with 10^6 PFU of SG-MCMV.
[c] ND, Not done.

The specific lysis was calculated by the formula (each release is measured in counts per minute): percent specific lysis = [(test release − spontaneous release)/(maximum release − spontaneous release)] × 100. Maximum release was determined in wells treated with 2 N NaOH. Spontaneous release was determined in wells added with unlabeled target cells instead of effector cells.

Antiasialo GM1 antibody. Rabbit antiasialo GM1 antibody (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was diluted 1:15 with sterile physiological saline. Mice were injected i.p. with 0.5 ml of the diluted antiasialo GM1 antibody at days 6 and 2 before MCMV infection.

Titration of viruses in organs. Weanling mice (eight mice per group) were inoculated i.p. with 10^6 PFU of SG-MCMV; 3 days later the liver and spleen were removed, and infectious viruses in the organs were titrated by using the supernatant fluid of a 10% (wt/vol) homogenate in Eagle MEM.

Mouse IFN. Mouse L-cell IFN, prepared from the supernatant fluid of an L-929 cell culture infected with Newcastle disease virus at a multiplicity of infection of 50 to 100, was kindly supplied by J. Imanishi, Kyoto Prefectural University of Medicine.

IFN assay. Serum IFN was assayed by the 50% plaque reduction method. Briefly, L-929 cells were seeded onto 35-mm polystyrene dishes (Corning Glass Works, Corning, N.Y.). After 5 to 6 h, when the cells attached to the dishes, the cells were treated with 0.1 ml of each two-dilution of test samples and authentic mouse IFN (positive control) or with nothing (untreated control) for an additional 24 h. The treated cells were washed with Eagle MEM and then inoculated with 100 PFU of the Indiana strain of vesicular stomatitis virus. After incubation for 2 days at 37°C under an atmosphere of 5% CO_2, the plaques were counted after staining with neutral red (0.005% in 1 ml of Eagle MEM per dish), which was added 1 day previously. IFN activity was expressed as the reciprocal of the highest serum dilution showing 50% plaque reduction.

TABLE 2. LD_{50} of SG-MCMV in BRM-treated mice

BRM[a]	Time of treatment[b]	LD_{50}[c]
None		6×10^5
OK-432	−3 and −1	$>5 \times 10^6$
OK-432	−1	$>5 \times 10^6$
PS-K	−3 and −1	$>5 \times 10^6$
PS-K	−3	2×10^6

[a] Six-week-old mice were injected i.p. with 0.02 mg of OK-432 per mouse or 10 mg of PS-K per mouse.
[b] Days before MCMV challenge.
[c] LD_{50} calculated by the Reed-Muench method.

TABLE 3. Effect of BRM on MCMV infection in suckling mice[a]

Group	Mortality[b] (%)	Body wt (g)[c]
Uninfected control	0/59 (0)	14.50 ± 2.10
OK-432-MCMV		
Saline	25/31 (81)	7.98 ± 3.87
0.02 mg	5/25 (20)[d]	13.06 ± 3.53
0.002 mg	12/23 (52)[e]	12.16 ± 4.18
PS-K-MCMV		
Saline	38/40 (95)	8.80 ± 2.26
1.0 mg	14/25 (56)[d]	12.82 ± 5.05
0.125 mg	13/28 (46)[d]	11.78 ± 4.08

[a] Suckling mice were injected with BRM within 24 to 48 h of birth and were infected 48 h later with 10^5 PFU of CC-MCMV. Control mice received i.p. physiological saline. Mice were examined daily until day 21 and were weighed on day 21.
[b] Number of mice dead/number of mice challenged.
[c] Mean ± standard deviation.
[d] $P < 0.005$ versus infected-saline control.
[e] $P < 0.10$.

RESULTS

Effect of BRM on MCMV infection in weanling mice. First, mice were treated once with either 0.02 mg of OK-432 or 10 mg of PS-K on various days before and after challenge with

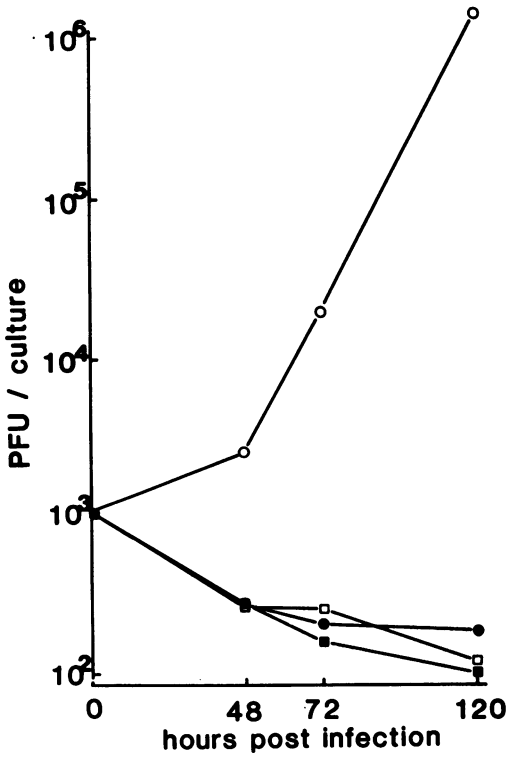

FIG. 1. Inhibition of the replication of SG-MCMV by peritoneal cells. SG-MCMV-infected MEF were untreated (○) or cocultivated with peritoneal cells from mice treated with OK-432 (■), PS-K (●), or saline (□). Peritoneal cell to MEF ratio was 50:1.

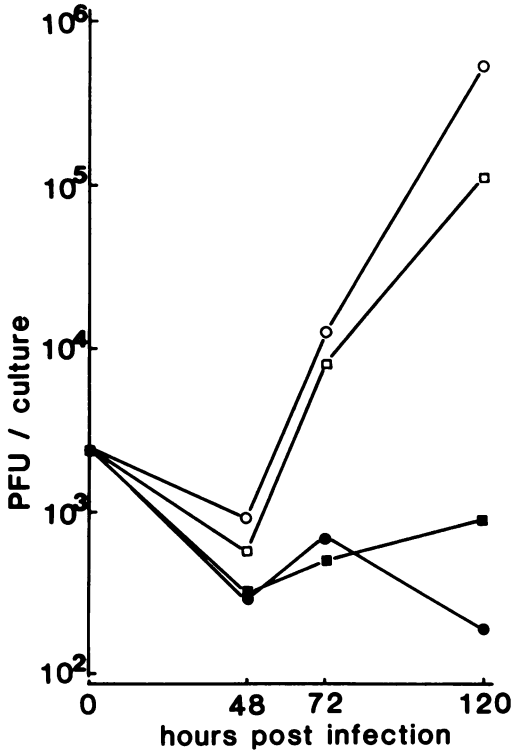

FIG. 2. Inhibition of the replication of SG-MCMV by spleen cells. SG-MCMV-infected MEF were untreated (○) or cocultivated with spleen cells from mice treated with OK-432 (■), PS-K (●), or saline (□). Spleen cell to MEF ratio was 200:1.

10^6 PFU, ca. 2 LD_{50}, of SG-MCMV. OK-432 and PS-K protected mice against lethal infection when administered i.p. on days 1 and 3 before infection, respectively (Table 1).

Second, the LD_{50} was compared between untreated and BRM-treated mice. The efficacy of single or double administrations was quantified by an increase in LD_{50} for BRM-pretreated mice (Table 2).

Effect of BRM on MCMV infection in suckling mice. Newborn mice were injected i.p. with two different doses of OK-432 and PS-K 2 days before challenge with 10^5 PFU of CC-MCMV. Both OK-432 and PS-K significantly decreased the mortality of mice (Table 3). When compared with the age-matched uninfected control mice, the MCMV-infected mice had retarded physical growth, as reported by Cruz and Waner (5). OK-432 and PS-K pretreatment improved the growth retardation of suckling mice caused by MCMV infection, although these results were statistically not significant (Table 3).

Lack of direct effect of BRM on MCMV and its replication. Both OK-432 and PS-K did not inactivate SG-MCMV or inhibit its replication and plaque formation in MEF at a concentration of 0.04 or 20 mg/ml, respectively, whether pretreated or posttreated (data not shown).

Inhibition of replication of MCMV by peritoneal and spleen cells. Replication of MCMV in MEF was completely inhibited by cocultivation with the peritoneal cells, mostly macrophages, from untreated as well as BRM-treated mice without disclosing the effect of BRM treatment (Fig. 1). Inhibition of MCMV replication by normal macrophages was compatible with other reports, such as those by Selgrade and Osborn (26) and Mims and Gould (18), which demonstrated the importance of macrophages in defense against MCMV infection.

TABLE 4. Effect of BRM on NK cell activity of spleen cells

In vivo treatment[a]		NK cell activity[b] (% specific lysis)	
BRM	SG-MCMV (PFU per mouse)	Expt 1	Expt 2
Saline	0	22.9	19.8
Saline	10^4	66.3	48.3
Saline	10^6	0	13.3
OK-432	0	34.7	28.1
OK-432	10^6	36.5	31.9
PS-K	0	38.7	36.5
PS-K	10^6	33.3	34.6

[a] BRM or saline was administered to mice on days 3 and 1 before challenge. The spleen cells were prepared from the treated mice at the time of challenge (uninfected mice) or on day 3 after challenge (infected mice).
[b] Effector to target cell ratio of 100:1.

In contrast, the spleen cells from BRM-treated mice, but not from untreated mice, interfered with replication of SG-MCMV when cocultivated with the infected MEF (Fig. 2). Thus, it was likely that the protective effect of OK-432 and PS-K was mediated by activation of the spleen cells.

NK cell activity of spleen cells. Activation of NK cells was examined with YAC-1 as target cells. NK cell activity of the spleen cells from mice infected with 10^4 PFU (sublethal dose) of SG-MCMV was greatly increased, but it was markedly inhibited by lethal infection with 10^6 PFU of SG-MCMV (Table 4).

Administration of OK-432 and PS-K augmented the NK cell activity of the spleen cells on the one hand and, on the other hand, protected impairment of the NK cell activity that was usually induced by lethal infection with SG-MCMV (Table 4).

Effect of BRM, antiasialo GM1 serum, or both on in vivo replication of MCMV. It has been shown previously that asialo GM1 is expressed on mouse NK cells (12), and treatment of NK cells with antiasialo GM1 antibody abolishes NK cell activity in vivo (9, 13). So, we examined the effect of antiasialo GM1 antibody on the replication of MCMV in the major target organs (liver and spleen) in combination with BRM treatment.

When mice were inoculated i.p. with 10^6 PFU of SG-MCMV, $10^{5.5}$ and $10^{5.7}$ PFU of MCMV were detected in the liver and spleen, respectively, of the saline-treated control mice on day 3 postinfection (Fig. 3). Treatment with antiasialo GM1 antibody alone increased the amount of MCMV replicated in those organs. A similar effect of antiasialo GM1 antiserum was recently published (3).

In contrast, BRM treatment remarkably suppressed the virus replication; namely, the amount of infectious virus recovered from the liver was significantly reduced by treatment with either OK-432 or PS-K ($P < 0.001$), and the amount in the spleen was significantly lower in the PS-K-treated mice ($P < 0.001$). However, when the mice were treated with antiasialo GM1 antibody concomitantly with OK-432 or PS-K, the amount of viruses in the organs completely returned to that of the saline-treated control mice (Fig. 3). Under such conditions, NK cell activity of the spleen cells was impaired, and the mice died, as did the saline-treated mice (data not shown).

Thus, antiasialo GM1 antibody nullified the protective effect of BRM, and death of mice due to MCMV infection

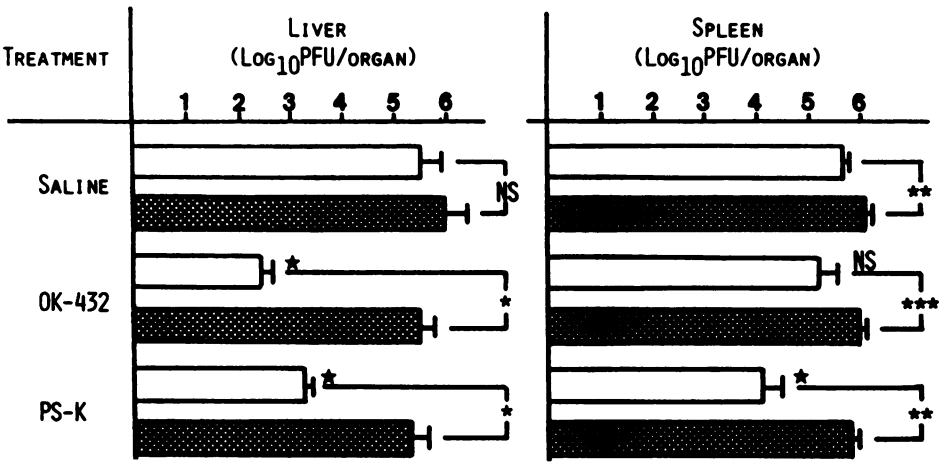

FIG. 3. Infectious viruses in the livers and spleens of mice treated with BRM, antiasialo GM1 antibody, or both. Groups, consisting of eight mice, received i.p. either saline or BRM on days 3 and 1 before infection. Half of the groups were further treated i.p. with antiasialo GM1 antibody on days 6 and 2 before infection in combination with saline or BRM. Infectious viruses in the organs were titrated at day 3 after infection. The bars in the histogram indicate the standard errors. Symbols: ☐, BRM or saline; ▩, BRM or saline plus antiasialo GM1 antibody; ★, $P < 0.001$ (compared with saline-treated control); *, $P < 0.001$; **, $P < 0.01$; ***, $P < 0.1$; NS, not significant.

correlated with replication of the virus in the liver and spleen, especially in the former.

Effect of mouse IFN on MCMV infection. Mouse IFN (5×10^4 IU per mouse) had no effect on the mortality and mean survival days of MCMV-infected mice when administered i.p. to weanling mice at various times ranging from 48 h before to 24 h after infection with 1×10^6 PFU of SG-MCMV (data not shown).

Lack of IFN induction by BRM treatment. It has been previously reported that OK-432, but not PS-K, induces IFN in DDI mice (17). However, no IFN was induced in ICR mice when tested at 12 and 24 h after a single injection with OK-432 or PS-K (Table 5). This discrepancy could be due to differences in the mice used, since IFN induction by OK-432 in spleen cell culture was shown to be dependent on the strain of mouse (25).

On the other hand, MCMV infection induced certain levels of IFN in untreated control mice. However, pretreatment of the mice with BRM on days 3 and 1 before virus challenge did not enhance the IFN induction in MCMV-infected mice (Table 5).

TABLE 5. Serum IFN levels in mice treated with BRM, MCMV, or both

BRM treatment	SG-MCMV (PFU per mouse)	Serum IFN titer[a] (U/ml)	
		12 h[b]	24 h[b]
OK-432	0	<20	<20
PS-K	0	<20	<20
None (saline)[c]	10^6	20	50
OK-432[c]	10^6	<20	<20
PS-K[c]	10^6	20	30

[a] IFN titers of pooled serum from seven mice per group.
[b] Time after BRM treatment or SG-MCMV challenge.
[c] Mice were treated i.p. with BRM or saline on days 3 and 1 before SG-MCMV challenge.

DISCUSSION

In this study, the protective effect of two BRM (OK-432 and PS-K) was examined in two systems of lethal infection: SG-MCMV versus weanling mice and CC-MCMV versus suckling mice. In either system, the BRM protected mice nonspecifically from lethal infection by MCMV. Then, experiments were done to elucidate the target organs of MCMV in lethal infection and the mechanism of BRM-induced protection.

As for the target organs, the BRM pretreatment of mice suppressed the replication of MCMV in the liver and, consequently, saved their lives. It has been previously shown that the titer of MCMV replicated in the liver of genetically susceptible mice is higher than that in the liver of genetically resistant mice (1, 16). In severe acute infection, 15-fold-fewer viruses were recovered from the livers of the resistant mice compared with the susceptible mice, whereas similar amount of viruses were recovered from the spleens of both strains of mice (1). In addition, when mice were protected specifically from lethal infection by immunization with attenuated MCMV (19) or *ts* mutants of MCMV (29), the livers of the mice were found to be free of MCMV infection (19, 29). Thus, involvement of the liver seems to be critical for mice in MCMV infection.

Host defense mechanisms against MCMV infection include neutralizing antibody (20, 22), cytotoxic T lymphocytes (23, 24, 28), macrophages (18, 26), antibody-dependent cell-mediated cytotoxicity (15, 22), NK cells (2, 8, 27), and INF (4). Since neutralizing antibody is detectable after 14 to 21 days (20, 22), it cannot be expected in acute infection. Such will also be the case for antibody-dependent cell-mediated cytotoxicity, which is first detectable on ca. day 10 (15). Cytotoxic T lymphocytes are detectable in the spleen between days 3 and 5 and peak between days 7 and 10 (23, 24). In our experiment, peritoneal cells from BRM-treated and untreated mice showed no difference in inhibition of the replication of MCMV in vitro, although the effector cells were not identified.

On the other hand, splenic NK cells can be stimulated within 10 h of MCMV infection, depending on virus dose and host genotype (2). The augmentation of NK cell activity correlates well with susceptibility of mice to MCMV infection (2). The augmentation of NK cell activity induced by MCMV infection is greater in athymic *nu/nu* mice than in euthymic *nu/+* littermates (2), and death occurs later in the

TABLE 6. Effect of BRM on the host-MCMV relationship

Condition		Parameter		Outcome
Mouse (treatment)	Challenge dose of MCMV	NK cell activity of the spleen cells	Virus replication in the liver	
ICR (none)	+	↑ ↑	+	Survival
ICR (none)	+++	↓	+++	Death
ICR (OK-432 or PS-K)	+++	↑	+	Survival
ICR (OK-432 or PS-K + antiasialo GM1 antibody)	+++	↓	+++	Death
Beige mutant (none)[a]	+++	↓	+++	Death
Susceptible strain (none)[a]	+	↑	+	Survival
Susceptible strain (none)[a]	++	↓	+++	Death
Resistant strain (none)[a]	+++	↑ ↑	++	Survival

[a] Adopted from references 1, 2, and 27.

former than in the latter (7). Although NK cell activation cannot by itself ensure mice protection against lethal MCMV infection, since *nu/nu* mice are more susceptible than *nu/+* mice, it will modify the course of MCMV infection, especially at its early stage. Splenic NK cell activity was significantly augmented by administration of OK-432 and PS-K. Especially, the two BRM supported NK cell activity after infection with lethal doses of MCMV, which impaired the activity in untreated mice. This correlated with virus titer in the liver and survival of the mice. Treatment of mice with antiasialo GM1 antibody also confirmed the role of the NK cell as an effector of BRM-induced resistance. The results obtained with antiasialo GM1 antiserum are compatible with those recently published by Bukowski et al. (3).

NK cells could be activated either directly by BRM or indirectly by BRM-induced IFN. In our system, IFN was neither induced in BRM-treated mice nor enhanced by BRM treatment in MCMV-infected mice. In addition, exogeneous IFN (mouse L-cell IFN) showed no effect on the course of MCMV infection. Thus, it is likely that BRM augment NK cell activity independently of IFN induction. In fact, it has been documented that the presence of anti-IFN antibody does not inhibit the cytotoxic activity of NK cells activated with OK-432 in an in vitro system (32).

Our conclusions are summarized in Table 6. There are some similarities between BRM-treated mice and genetically resistant mice. Susceptibility of suckling mice to CC-MCMV was reduced by BRM treatment. This may also be attributable to NK cell activity, although further analysis was not done for technical reasons. In fact, NK cell activity was deficient in suckling mice and developed with age, in correlation with age-dependent resistance of mice to CC-MCMV (K. Hayashi, personal communication).

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