Cytokine Production in the Immune Response to Murine Gammaherpesvirus 68

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Received 30 October 1995/Accepted 26 January 1996

Cytokine profiles were determined following intranasal infection of C57BL/6J mice with murine gammaherpesvirus 68 (MHV-68). Spleen, mediastinal, and cervical lymph node cells from infected mice produced high levels of interleukin 6 (IL-6) and gamma interferon (IFN- γ) and lower levels of IL-2 and IL-10 following in vitro restimulation. Little or no IL-4 or IL-5 was detected. Cytokine production was generally maximal at 10 days after infection, correlating with viral clearance from the lung, although significant levels were seen as early as 3 days after administration of the virus. In vitro infection of naive splenocytes induced B-cell-dependent secretion of IL-6 and IL-10, whereas IFN- γ and IL-2 were produced only by cells that had been primed in vivo. Depletion of B lymphocytes from primed splenocyte populations did not, however, abrogate IL-6 and IL-10 production. Highly purified immune T cells made IL-6, IL-10, and IFN- γ following in vitro restimulation with MHV-68. Thus, IL-6 and IL-10 are components of both the acquired and the innate host response. These cytokines have potential roles in the establishment and maintenance of persistent infection.

Murine gammaherpesvirus 68 (MHV-68) is a naturally occurring rodent pathogen (1) which, from the structure of the viral genome and limited sequence data, appears to be closely related to Epstein-Barr virus (EBV) and herpesvirus saimiri (8). Intranasal administration of MHV-68 results in acute productive infection of lung alveolar epithelial cells and a persistent latent infection in B lymphocytes, the spleen being a major reservoir of latent virus (28, 29). Infectious virus can be recovered from the lungs of BALB/c mice (28) and C57BL/6 mice (1a) for 10 to 13 days after infection. Immune CD8⁺ T cells, but not CD4⁺ T cells, are essential for clearance from the respiratory tract and recovery from the acute phase of the infection. However, virus elimination from the respiratory mucosa is slightly delayed in CD4-depleted mice and splenomegaly, which is characteristic of this infection, is reduced or absent (9). Like EBV, MHV-68 is associated with lymphoproliferative disease (27). Although there has been no previous analysis of the cytokine response to MHV-68, gamma interferon (IFN- γ) plays a role in the clearance of several different herpesviruses (17, 22, 24), while interleukin 6 (IL-6) and IL-10 have been implicated in the establishment and maintenance of persistent EBV infections (20, 32, 33). Analyzing cytokines during MHV-68 infection indicates that this virus induces a characteristic profile, reflecting aspects of both the specific and nonspecific host response.

MHV-68 virus was obtained from A. Nash, and stocks were grown in owl monkey kidney (OMK) cells (ATCC CRL 1556). Female C57BL/6J mice, ages 6 to 10 weeks (Jackson Laboratory, Bar Harbor, Maine), were infected intranasally with 4,000 PFU. This led to a 5- to 10-fold increase in cellularity in the mediastinal lymph node (MLN), which drains the lower respiratory tract (Fig. 1). Maximal cell numbers were observed 10 to 15 days after infection, the time at which virus is cleared from the lung (1a). Similar increases were seen in the cervical

lymph nodes (CLN) and spleen (Fig. 1), although cell numbers peaked slightly later (day 15 after infection). The MLN results are typical for localized infections with respiratory viruses. However, the dramatic increase in the cellularity of the spleen is not seen for the influenza A viruses (20a). Cell numbers in the lymphoid organs had decreased by 20 to 70 days after infection (Fig. 1).

Since increases in cellularity of the lymphoid tissue might be associated with changes in lymphocyte subset composition, which could in turn influence cytokine profiles, the relative proportions of T-cell subsets, B cells, and NK cells in the spleen and lymph nodes of MHV-68-infected mice were analyzed. Transient changes were seen in the relative proportions of the different lymphocyte subsets over the 70-day period analyzed (Fig. 2). The ratio of CD4⁺ to CD8⁺ T cells was decreased at 6 to 10 days after infection for the MLN and at 10 to 30 days after infection for the spleen (Fig. 2). Comparison of CD4/CD8 ratios in the spleen at days 13 and 15 with those for age-matched naive controls showed that this change was statistically significant ($P = 0.0025$; Student's t test). Although T-cell/B-cell ratios appeared to vary at some time points, the differences were not statistically significant. Very few γ/δ T cells were seen in any of the tissues studied, while the proportion of NK cells in the spleen remained constant at 4.0 to 5.5% at days 0, 10, and 15 after infection (data not shown). The distribution of lymphocyte subsets in the CLN closely resembled that in the MLN (data not shown). The observed changes in the prevalence of the various lymphocyte subsets were small and are unlikely to have greatly influenced the cytokine profiles.

Cytokine production was analyzed for culture supernatants from cells that had been restimulated in vitro with virus-infected irradiated antigen-presenting cells. Cells from the MLN, CLN, or spleens of infected animals produced substantial amounts of IL-6, IL-10, and IFN- γ when restimulated in vitro (Fig. 3), while IL-4 and IL-5 were not found at any stage (data not shown). Maximal cytokine production was seen 10 days after infection, immediately before virus is cleared from the lungs, suggesting a potential role in this process. Low, variable

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FIG. 1. Cell numbers in the MLN, CLN, and spleen were determined at intervals following intranasal infection of mice with MHV-68. Single-cell suspensions were prepared from lymphoid tissue from groups of 3 to 5 terminally anesthetized exsanguinated mice, and viable cell counts were determined by trypan blue exclusion. Data are expressed as means plus standard errors of the means for three to five separate experiments at each time point.

amounts of IL-2 were detected predominantly in MLN culture supernatants (Fig. 3). Minimal IL-2 production could reflect either consumption or a defect induced by the virus. The latter has been recognized following infection with lymphocytic choriomeningitis virus and human herpesvirus 6, both of which reduce the capacity of T cells to produce IL-2 and to proliferate following stimulation with mitogens *in vitro* (4, 11). Although some CD4⁺ T cells are infected with lymphocytic choriomeningitis virus and human herpesvirus 6, the frequency is generally too low to explain the lack of response. It is not known whether MHV-68 infects T cells *in vivo*. However, one study indicated that MHV-68 did not replicate in a T-cell line (30) while fractionation of lymphoid tissue from infected mice showed that the majority of virus was in the B cells (29). Despite the low level of IL-2 production after restimulation with virus, cytokine responses of mitogen-stimulated splenocytes from MHV-68-infected animals appeared normal (Fig. 4). These results did not indicate a general immunosuppressive effect of the virus.

Lack of IL-4 and IL-5 production could be due to the high level of IFN- γ present, since this cytokine can inhibit the expansion of Th2 cells (12). Alternatively, since low levels of IL-4 produced early in immune responses may be necessary to

prime cells for IL-4 and IL-5 production, the failure of MHV-68 to trigger detectable IL-4 secretion early may limit the subsequent development of IL-4- and IL-5-secreting cells (15, 31).

Cytokine levels were also examined in bronchoalveolar lavage fluid obtained by washing the lungs of MHV-68-infected mice via the trachea three times with 1 ml of phosphate-buffered saline–1% bovine serum albumin. Bronchoalveolar lavage fluid was sampled at 6, 10, 15, and 30 days after infection and was concentrated 10- to 20-fold by using Amicon Centriprep 10 concentrators, prior to analysis of cytokines by enzyme-linked immunosorbent assay (ELISA). Even after concentration, cytokines were detected only at days 10 and 15 after infection. The levels obtained in two separate experiments were as follows: IL-2 (day 10), 0.0 and 1.3 U/ml; IL-2 (day 15), 0.0 and 0.0 U/ml; IL-6 (day 10), 16 and 22 U/ml; IL-6 (day 15), 0.0 and 0.0 U/ml; IL-10 (day 10), 1.0 and 1.7 ng/ml; IL-10 (day 15), 0.9 and 1.6 ng/ml; IFN- γ (day 10), 3.2 and 1.2 U/ml; IFN- γ (day 15), 0.0 and 0.5 U/ml. No IL-4 or IL-5 was detected. Thus, the cytokine profile was similar to that obtained after *in vitro* restimulation of lymph node or spleen cells. Cytokine levels in the lungs peaked just prior to viral clearance, suggesting a potential role in this process.

FIG. 2. Lymphocyte subsets in the MLN and spleen during the immune response to MHV-68. Lymphocytes were stained with phycoerythrin or fluorescein-conjugated monoclonal antibodies as previously described (21) except that cells were fixed in 1% paraformaldehyde (Ted Pella Inc., Redding, Calif.) after staining. All antibodies were purchased from PharMingen (San Diego, Calif.). The detection limit was less than 1% on the basis of staining with isotype-matched controls. Results are expressed as mean percent positive cells (determined by flow cytometry) for two to five separate experiments at each time point plus standard errors of the means (for three or more datum points).

FIG. 3. Cytokine production during MHV-68 infection. MLN, CLN, or spleen cells were resuspended at a final density of 2×10^6 cells per ml in RPMI-1640 supplemented with 10% fetal calf serum, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 1 mM glutamine, and 10 μ g of gentamicin per ml and restimulated in vitro with virus-infected (multiplicity of infection = 0.01), irradiated (3,000 rads) splenocytes at a final density of 2×10^6 cells per ml as antigen-presenting cells. Supernatants were harvested after 24, 48, 72, or 96 h and stored at -80°C prior to assaying cytokines by sandwich ELISA as previously described (21). Peak levels are reported. Maximal cytokine production was seen at 24 to 48 h for IL-2 and 72 h for all other cytokines. The detection limit was below 0.4 ng/ml for IL-10 and below 1 U/ml for the other cytokines. Data are means for two to five separate experiments at each time point plus standard errors of the means (for three or more datum points) and are expressed as units per milliliter for IL-2, IL-6, and IFN- γ and as nanograms per milliliter for IL-10. No IL-4 or IL-5 was detected. Cells from MLN, CLN, or spleens of mice mock-infected with extracts of uninfected OMK cells produced a range of cytokines similar to that produced by naive cells (data not shown). Unstimulated cultures and those restimulated with influenza virus-infected irradiated antigen-presenting cells generally failed to produce cytokines, although occasionally cells taken at days 10 and 13 produced low levels of IFN- γ (data not shown). MHV-68-infected irradiated antigen-presenting cells did not produce detectable amounts of cytokine.

FIG. 4. Response of splenocytes from MHV-68-infected animals to mitogens. Splenocytes from naive or MHV-68-infected animals were resuspended at 2×10^6 cells per ml and stimulated with 2 μ g of concanavalin A (Con A) (Sigma) per ml. Control cultures were untreated. Supernatants were harvested for cytokine ELISA as described in the legend to Fig. 3 at 24 to 72 h.

Virus-induced cytokine production in the spleen and lymph nodes generally decreased at later time points (30 to 70 days after infection), although significant levels of IFN- γ , IL-6, and IL-10 were still detected following in vitro culture (Fig. 3). Substantial amounts of IL-6 and IL-10 were also found following restimulation (Fig. 3, day 0) or direct infection (Table 1) of naive spleen and lymph node cells, whereas IFN- γ and IL-2 induction required prior in vivo priming. Incubation of splenocytes with infectious influenza A virus (HKx31 strain), boiled MHV-68, or a mock virus preparation containing uninfected OMK cell extracts failed to stimulate the cells to make IL-6 or IL-10 (Table 1), indicating that induction of these cytokines requires live or native MHV-68. This IL-6 and IL-10 production is not common to all cell types, however, as NIH 3T3 fibroblasts could be productively infected (as plaques formed in monolayer cultures), but they did not produce either cytokine (Table 1). Depletion of B cells with Dynal beads abrogated IL-6 and IL-10 production, whereas removing CD4 $^{+}$ and CD8 $^{+}$ T cells had no effect (Table 1). Similarly, when naive spleen cells were separated by fluorescence-activated cell sorting (FACS) into B- and T-cell subsets, only the B-cell fraction produced IL-6 on subsequent in vitro infection (Table 1). There was some reduction in the amount of IL-6 produced after cell sorting, perhaps reflecting damage to the cells during passage through the flow cytometer. IL-10, which was found at lower levels than IL-6 before sorting, was no longer detectable. It is not clear why MHV-68-infected B cells produce IL-6 and IL-10 whereas infected fibroblasts do not. This may be due to

TABLE 1. Cytokine production by unprimed splenocytes^a

Stimulus	Cell type	n ^b	Concn of cytokine produced ^c	
			IL-6 (U/ml)	IL-10 (ng/ml)
None	Splenocytes	3	0 ± 0	0 ± 0
MHV-68 ^d	Splenocytes	3	29.1 ± 3.9	4.7 ± 0.6
Mock infection ^e	Splenocytes	2	0	0
Boiled virus ^f	Splenocytes	2	0	0
Influenza virus ^g	Splenocytes	2	0	0
MHV-68	NIH 3T3 fibroblasts	2	0	0
MHV-68	B-cell-depleted splenocytes ^h	3	0.5 ± 0.3	0.5 ± 0.3
MHV-68	CD4 + CD8 T-cell-depleted splenocytes ^h	1	35.1	5.3
MHV-68	Splenic T cells ⁱ	2	0	0
MHV-68	Splenic B cells ⁱ	2	17.4	0
MHV-68	Unseparated splenocytes ⁱ	2	22.5	3.3

^a Cells were treated in vitro for 24 to 72 h, after which supernatants were harvested for cytokine analysis by ELISA (see the text and the legend to Fig. 3 for details).

^b n, number of experiments.

^c Data are means ± standard errors of the means (where three experiments were done). No IL-2, IL-4, IL-5, or IFN-γ was detected.

^d Splenocytes were infected with virus at a multiplicity of infection of 0.01 in complete medium: RPMI 1640 containing 10% fetal calf serum, 10 mM HEPES, 1 mM glutamine, and gentamicin (10 μg/ml). Infection was done at a cell density of 5 × 10⁷/ml for 2 h at 37°C, after which the culture was diluted with complete medium to a cell density of 4 × 10⁶/ml.

^e Mock infections were done as described above but with an extract of uninfected OMK cells.

^f MHV-68 virus was boiled for 10 min, and an equivalent multiplicity of infection of 0.01 was used.

^g Splenocytes were infected with influenza virus A/HKx31 as described for MHV-68.

^h Splenocytes were depleted of B220⁺ B cells or CD4⁺ and CD8⁺ T cells by staining with biotinylated anti-B220 (clone RA3-682), anti-CD4, or anti-CD8 (clones 57-6.7 and RM4-4) at a concentration of 5 μg/ml prior to removal of stained cells by using streptavidin-coated Dynal Beads (Dynal, Lake Success, N.Y.). Biotinylated monoclonal antibodies were purchased from PharMingen (San Diego, Calif.). The percent contamination was 2.8 ± 0.3% for B cells (determined by staining with goat anti-mouse immunoglobulin (Jackson ImmunoResearch Laboratories, West Grove, Pa.) and 3.5% for the population depleted of CD4⁺ and CD8⁺ cells (determined by staining with phycoerythrin-conjugated anti-α/β T-cell receptor, clone H57-597; PharMingen).

ⁱ Naive splenocytes were stained with fluorescein isothiocyanate-conjugated anti-B220 and phycoerythrin-conjugated anti-α/β T-cell receptor (PharMingen) prior to sorting into T- and B-cell populations by using a FACStar Plus (Becton Dickinson, Mountain View, Calif.). Sorted populations were >98% pure and were infected with MHV-68 as described above. Unseparated cells were stained but not sorted.

lack of specific transcription factors, failure to produce essential cofactors, or production of inhibitory factors.

Spleen and lymph node cells from infected animals appeared to produce greater amounts of IL-6 and IL-10 after in vitro restimulation than cells from naive mice (Fig. 3). This suggested that cell types in addition to B cells (possibly T cells) might be producing cytokines during infection. Therefore, lymphocyte subsets were depleted in vitro from the spleens of animals infected 13 to 15 days earlier with MHV-68, by using Dynal beads to determine which cell types produced the various cytokines. Depletion of B cells failed to abolish IL-6 and IL-10 production (Table 2), as it did in the naive spleen cell populations (Table 1), despite the fact that the percentage of contaminating B cells was similar in each case. The levels of both IL-6 and IL-10 were reduced, however, although there was relatively little change in the amount of IFN-γ. This suggests that B cells make some, but not all, of the IL-6 and IL-10 during infection and do not make IFN-γ. Expansion of the

contaminating B-cell population is an unlikely explanation for the residual IL-6 and IL-10 production, as the percentage of B cells was only 4% after culture compared with 48% in an undepleted culture. Apparently, other cell types, in addition to B cells, produce IL-6 and IL-10 during MHV-68 infection. This was confirmed by comparing cytokine production by restimulated T- and B-cell populations purified by FACS (Table 2). Although there did appear to be some loss in activity due to staining and/or sorting, it was clear that both T- and B-cell populations produced IL-6 and IL-10 following restimulation in vitro while IFN-γ production was confined to α/β T-cell-receptor-positive cells.

Thus, IL-2 and IFN-γ production appear to reflect a specific acquired response to the virus, whereas the induction of IL-6 and IL-10 occurs (in part) via an innate mechanism but still depends on the presence of live or native virus. The nonspecific production of IL-6 and IL-10 could represent an early host defense mechanism, as both cytokines have been implicated in the growth or activation of cytotoxic effectors (3, 16, 26). Alternatively, induction of these cytokines may be an adaptive feature of MHV-68 enabling it to establish and maintain a persistent infection. There is evidence for the latter in EBV infection. IL-6 stimulates proliferation of EBV-infected B cells and increases tumorigenicity of EBV-infected lymphoblastoid cells (33, 34), suggesting that this cytokine acts as an autocrine growth factor (34). High concentrations of IL-6 can also inhibit killing of EBV-infected cells by NK cells (33). It is intriguing that sequences from a novel human gammaherpesvirus have recently been detected in Kaposi's sarcoma tissue (2). As IL-6 is a growth factor for Kaposi's sarcoma cells (19), induction of this cytokine by the virus might play a role in tumorigenesis. Furthermore, IL-10 is a growth factor for B cells (13) which could also facilitate viral persistence. Infection of B cells with EBV results in expression of BCRF-1 (a viral homolog of IL-10) in 6 to 8 h and induction of cellular IL-10 by 24 to 48 h (20). Both viral and cellular IL-10 can inhibit macrophage activation and antigen presentation (6). The IL-10 produced during MHV-68 infection could be of viral or cellular origin, although a viral homolog of IL-10 has not yet been identified in MHV-68.

Both IL-2 and IFN-γ are likely to be involved in host defense, although they can also support B cell growth (18, 23, 25). Proliferation and activation of cytotoxic effectors in vitro is

TABLE 2. Cytokine production by splenic lymphocyte subsets 13 to 15 days after infection of mice with MHV-68

Cell population ^a	n ^b	Concn of cytokine produced ^c		
		IL-6 (U/ml)	IL-10 (ng/ml)	IFN-γ (U/ml)
Undepleted	4	44.8 ± 5.5	7.4 ± 0.8	21.3 ± 4.0
B-cell depleted	4	16.6 ± 1.6	4.9 ± 1.3	16.5 ± 3.9
Mock depleted	4	37.9 ± 5.1	7.0 ± 1.2	23.5 ± 4.6
B cells	3	10.8 ± 4.6	1.0 ± 0.3	0.0 ± 0.0
T cells	3	3.1 ± 1.0	2.5 ± 0.8	14.9 ± 4.6
B + T cells	3	44.6 ± 24.6	3.1 ± 1.0	32.6 ± 15.4
Unseparated	3	19.0 ± 7.0	3.6 ± 1.0	43.5 ± 18.0

^a Spleen cells from infected animals were depleted of lymphocyte subpopulations or sorted into T- and B-cell fractions as described in Table 1 and restimulated in vitro as described in the legend to Fig. 3. The percentage of contaminating cells in the B-cell-depleted population was 1.3 to 6.1%. FACS-separated populations were >98% pure. Unseparated cells were stained but not sorted. Undepleted cells were neither stained nor sorted. Mock depletions were carried out with biotinylated isotype-matched control antibodies.

^b n, number of experiments.

^c Supernatants were harvested for cytokine analysis by ELISA as described in the legend to Fig. 3. Data are means ± standard errors of the means.

enhanced by IL-2 (21), although the role of this lymphokine *in vivo* is currently unclear (14). Effective elimination of murine cytomegalovirus, pseudorabies virus, and herpes simplex virus in mice is dependent on IFN- γ (17, 22, 24). This cytokine also plays a part in cytotoxic T-cell, NK cell, or macrophage activation (5), in upregulating the expression of major histocompatibility complex and adhesion molecules (7, 35), in facilitating lymphocyte activation and recruitment, and in antibody production, particularly production of the immunoglobulin G2a isotype (10). Thus, the cytokines produced during MHV-68 infection may be important in both viral pathogenesis and host defense. Analyzing mice that lack functional genes for these cytokines should provide further insights into the roles of these proteins.

This work was supported in part by grants CA 21765, AI29579, and CA 09346 from the National Institutes of Health and by the American Lebanese and Syrian Associated Charities.

We are grateful to Roseann Lambert, Mahnaz Paktinat, and Jim Houston for assistance with flow cytometry and to Vicki Henderson for help in the preparation of the manuscript.

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