JOURNAL OF VIROLOGY, Nov. 1990, p. 5383–5388
0022-538X/90/115383-06$02.00/0

Vol. 64, No. 11

In Vivo Polyclonal B-Lymphocyte Activation Elicited by Murine Viruses

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Received 30 March 1990/Accepted 27 July 1990

Viruses such as lactate dehydrogenase-elevating virus and adenovirus induce in vivo a polyclonal activation of murine B lymphocytes, followed by a marked increase in the production of immunoglobulin G2a (IgG2a). The role of T lymphocytes in this phenomenon was studied by injection of an anti-CD4 monoclonal antibody able to inhibit the T-helper function. This treatment profoundly depressed the production of IgG2a, whereas it had no effect on the proliferation of B cells. Activated B cells obtained from such infected and treated mice remained able to produce various immunoglobulin isotypes after exposure to an appropriate stimulus. In particular, gamma interferon, which is known to be secreted after viral infection, induced the production of IgG2a. These observations support the hypothesis that the influence of viruses on the switch of immunoglobulins is mediated by T-helper lymphocytes.

Production of large amounts of immunoglobulins largely restricted to the immunoglobulin G2a (IgG2a) subclass is observed in mice infected with various types of viruses (1, 8, 10, 20). Experiments with adenovirus and lactate dehydrogenase-elevating virus (LDV) indicated that only a small fraction of these immunoglobulins (<5 to 10%) were antiviral antibodies and suggested that most of them resulted from a polyclonal activation of B lymphocytes (8, 10). Although a role of these immunoglobulins in the defense of the host cannot be excluded, virally induced polyclonal activation could result in autoimmune reactions and immunodeficiency (1, 20; C. J. Pfau, personal communication).

In this work, we studied in further detail the activation of spleen cells induced by infection with adenovirus and LDV. We investigated the role of T-helper cells by using a monoclonal anti-CD4 antibody (MAb) able to inhibit T-dependent immune responses in vivo (7). This treatment strongly depressed the production of IgG2a but did not affect the proliferation of B lymphocytes. We also tested the ability of different factors to restore the secretion of various immunoglobulin isotypes by such virally activated B cells from T-depleted animals.

MATERIALS AND METHODS

Mice. Specific-pathogen-free female CBA/Ht and CBA/Rij mice were purchased from the Ludwig Institute for Cancer Research and used at the age of 8 weeks.

Viruses. Mice were infected by intraperitoneal injection of approximately 2×10^7 50% infectious doses (ID_{50}) of LDV (Riley strain; from the American Type Culture Collection, Rockville, Md.) (10) or approximately 10^7 ID_{50} of the FL strain of adenovirus. The LDV infection was checked by assay of the lactate dehydrogenase activity in plasma, and the adenovirus infection was checked by conventional enzyme-linked immunosorbent assay (ELISA).

Antibody treatment. Ascitic fluid (400 to 500 μl) containing anti-Thy1 MAb (dilution 1:3 of A6703A8, an IgG2b anti-Thy1.2 MAb from an AKR mouse), anti-CD4 MAb (GK1.5, made available by F. W. Fitch and obtained through the courtesy of H. R. MacDonald) (11), or purified antibody was injected intraperitoneally. After GK1.5 treatment, flow cytometry analysis with another anti-CD4 MAb which did not compete with GK1.5, RL1724 (5), usually showed a nearly complete disappearance of T-helper lymphocytes. Anti-gamma interferon (anti-IFN-γ) was injected as ascitic fluid (up to 1 ml) containing either F3 (16), R4-6A2 (American Type Culture Collection, Rockville, Md.) (30), or XMG-1.2 MAb (kindly provided by T. Mosmann, DNAX, Palo Alto, Calif.) (6) or purified MAb (up to 1 mg).

Spleen cell cultures. As described previously (10), after centrifugation through a fetal calf serum (FCS) cushion, 25×10^6 spleen cells were cultured for 24 h in 5 ml of Iscove medium containing 10% FCS and supplemented with 0.24 mM L-asparagine, 0.55 mM L-arginine, 1.5 mM L-glutamine, and 0.05 mM 2-mercaptoethanol. For stimulation of lymphocytes from T-depleted infected animals, 10^6 spleen cells were cultured in 1 ml of the same medium for 5 days in the presence of lipopolysaccharide (LPS) from *Escherichia coli* (25 μg/ml).

Response to lymphokines was analyzed with B lymphocytes purified by panning on petri dishes coated with goat anti-mouse immunoglobulin antibodies (3 μg/ml) and bovine serum albumin (3 μg/ml), after elimination of macrophages by a preliminary incubation in tissue culture dishes. B cells (10^6) were incubated for 6 to 8 days in 1 ml of supplemented Iscove medium in the presence of LPS (20 μg/ml), with serial doses of murine recombinant interleukin-4 (rIL-4; produced in baculovirus; courtesy of R. Devos and W. Müller) or murine recombinant IFN-γ (rIFN-γ; kindly provided by W. Fiers). In the latter case, cells were washed after 2 days and incubated for 6 days with fresh medium containing LPS but no IFN-γ.

Immunoglobulin assay. IgG1, IgG2a, and IgG2b were assayed in spleen cell supernatants by inhibition ELISA (8) or by direct ELISA, the binding of IgG subclasses to

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TABLE 1. Spleen cell proliferation after adenovirus infection[a]

Adenovirus infection	Day p.i.	Mean [^{3}H]thymidine incorporation (cpm) ± SE
−		1,333 ± 143
+	4	4,246 ± 129
+	7	5,979 ± 2,088
+	10	21,500 ± 1,476
+	14	13,191 ± 1,277

[a] Proliferation was measured by [^{3}H]thymidine incorporation by 250,000 spleen cells obtained from CBA/Ht mice at different times after intraperitoneal infection with adenovirus. Results are expressed for groups of five animals.

nonsolubilized isotype-specific rabbit antibodies being measured with peroxidase-labeled goat anti-mouse immunoglobulin antibody (Kirkegaard & Perry Laboratories, Gaithersburg, Md.). For IgG3, plates coated with a rat anti-mouse IgG3 MAb (2E.6; American Type Culture Collection, Rockville, Md.) were incubated with serial dilutions of supernatants, followed by biotinylated rat anti-mouse IgG3 MAb and peroxidase-conjugated avidin. Standards were MAbs of the appropriate isotype.

Thymidine incorporation. Spleen cells (250,000) were incubated for 5 to 6 h at 37°C in 200 μl of Iscove medium supplemented as described above and containing 0.5 μCi of [*methyl*-^{3}H]thymidine (specific activity, 25 Ci/mmol; Amersham International, Amersham, U.K.). The cells were counted for radioactivity in an LS 3801 liquid scintillation system (Beckman Instruments Inc., Palo Alto, Calif.).

Flow cytometry. After lysis of erythrocytes in 0.83% NH_4Cl, spleen cells were incubated for 45 min at 4°C in HAFA buffer (137 mM NaCl, 5 mM KCl, 0.4 mM $MgSO_4$, 0.3 mM $MgCl_2$, 5 mM glucose, 4 mM $NaHCO_3$, 1 mM EDTA, 1 mM phosphate, 20 mM NaN_3, 100 U of penicillin per ml, 100 μg of streptomycin per ml [pH 7.4], supplemented with 3% FCS) with fluoresceinated or biotinylated MAbs reacting with different surface antigens, followed by fluoresceinated streptavidin. After fixation in 0.62% paraformaldehyde, fluorescence and Coulter volume were analyzed with an ATC3000 flow cytometer (ODAM, Wissembourg, France). Incorporation of 7-amino-dactinomycin was measured as described by others (25).

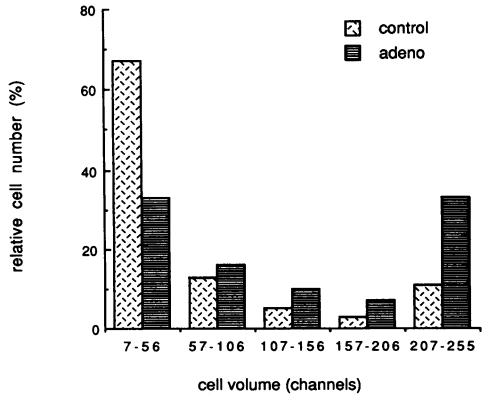

FIG. 1. Spleen cell volume after adenovirus infection. Coulter volume of pooled spleen cells from five control or infected (adeno) CBA/Ht mice at day 10 p.i. was measured by flow cytometry. Results, expressed on an arithmetical scale divided into 255 channels, are presented for clarity as percentage of total cell number in groups of approximately 50 channels. The absence of aggregated cells was separately checked by conventional optic microscopy.

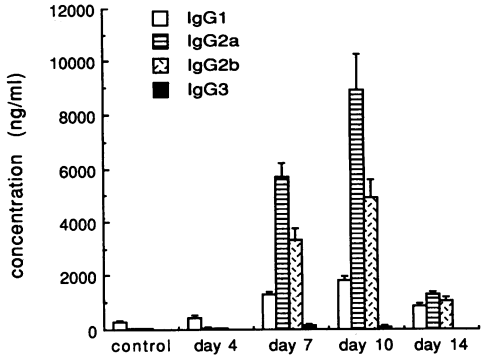

FIG. 2. IgG subclass production by spleen cells after adenovirus infection. IgG subclass levels were measured by ELISA in supernatants of spleen cell cultures initiated at different times after infection of CBA/Ht mice. Results are shown for groups of four to five animals (mean ± SE).

RESULTS

B-lymphocyte activation. Spleen cell proliferation was assessed by the thymidine incorporation test at different times after infection with adenovirus. An important proliferation started at day 4 and peaked at day 10 postinfection (p.i.) (Table 1). This proliferation correlated with an increase in the volume of a significant proportion of cells, as shown by flow cytometry (Fig. 1). Typing of these blastic cells with MAbs indicated that approximately 67% of them were B lymphocytes. Interestingly, the size of some T lymphocytes was also increased, but the proportion of T cells in the population with an enlarged cellular volume (14%) was smaller than in the total cell population (31%). In addition, enhanced labeling with 7-amino-dactinomycin was observed in 11% of spleen cells from infected animals, including 13% of B lymphocytes but only 5% of T cells, compared with 3% of spleen cells from control mice (data not shown). These observations indicated that adenovirus is a potent in vivo activator of murine B lymphocytes.

As expected from the previously reported increase in serum IgG2a and, to a lesser extent, IgG2b levels (8), the production of these isotypes by spleen lymphocytes was dramatically enhanced during adenovirus infection (Fig. 2). This effect was slightly delayed in comparison to the cell proliferation, but reached its maximum similarly at day 10 p.i.

LDV has also been shown to induce a considerable in vivo activation of spleen cells, characterized by an increase in thymidine incorporation that reaches a maximum at 4 days p.i. and by the production of large amounts of non-virus-specific IgG2a (3, 10, 21, 24). Flow cytometric analysis of

TABLE 2. Spleen cell proliferation after adenovirus infection and anti-CD4 treatment[a]

Adenovirus infection	Anti-CD4 treatment	Mean [^{3}H]thymidine incorporation[b] (cpm) ± SE
−	−	1,109 ± 264
+	−	17,244 ± 578
+	+	20,857 ± 4,108

[a] CBA/Ht mice treated with anti-CD4 MAb GK1.5 received 400 μl of ascitic fluid on days 0 and 5 p.i. as indicated.

[b] Thymidine incorporation by 250,000 spleen cells was measured 10 days p.i. Results are shown for groups of five animals.

TABLE 3. Proportion of B and T lymphoblasts after adenovirus infection and anti-CD4 treatment[a]

Adenovirus infection	Anti-CD4 treatment	% Blastic cells[b]		
		Total splenocytes	B lymphocytes	T lymphocytes
−	−	2	4	1
+	−	11	17	4
+	+	10	13	4

[a] CBA/Ht mice treated with anti-CD4 received 400 μl of GK1.5 ascitic fluid on days 0 and 5 p.i.

[b] Blastic cells were arbitrarily defined by their Coulter volume measured in flow cytometry (corresponding to the last column, channels 207 to 255, in Fig. 1). The proportion of B and T lymphoblasts is the ratio of blastic over total B and T cells.

spleen cells from mice infected with this virus indicated that a large proportion of activated cells were B lymphocytes (data not shown).

Role of CD4 cells. The T-cell dependence of virally induced spleen cell activation was assessed by injections, during the infection, of an antibody able to inhibit T-helper lymphocytes. This treatment had no effect on the proliferation of spleen cells after adenovirus infection (Table 2). Flow cytometry confirmed that a relatively constant proportion of B lymphocytes (approximately 15%) were activated in the animals treated or not treated with the anti-CD4 MAb (Table 3). Some activated T lymphocytes were observed in both groups of infected mice. Contrasting with the B-lymphocyte proliferation, the adenovirus-induced production of IgG was dramatically reduced by the suppression of functional T-helper cells (Fig. 3).

Similar results were obtained after LDV infection. Anti-T-helper treatment had no detectable effect on thymidine incorporation by spleen cells (Fig. 4A) but, in contrast, inhibited most of the virus-induced IgG2a production (Fig. 4B). This decrease in IgG2a as well as in IgG2b in anti-CD4-treated mice did not correlate with any modification in the level of the other IgG subclasses, nor of IgM or IgA (Table 4). The kinetics of this suppression is shown in Fig. 5. A single injection of MAb GK1.5 as late as 4 days p.i. still resulted in inhibition of IgG2a secretion, suggesting that T-helper cell activity was needed when the proliferation of B cells was maximal (10) in order to induce them to secrete immunoglobulins.

IgG production by spleen cells from CD4-depleted infected mice. Whether activated B lymphocytes from CD4-depleted infected mice were still capable of secreting various immunoglobulin isotypes was investigated by cultures of those cells in the presence of stimulating factors. LPS preferentially induced an IgG3 response, comparable to that obtained with spleen cells from control animals (Table 5). IgG1 predominated when purified B lymphocytes from T-helper-depleted LDV-infected mice were cultured with rIL-4 in the presence of LPS, quite similarly to what has been reported for B cells from normal animals (32) (shown in Fig. 6 for a typical experiment). Finally, IFN-γ induced some production of the IgG2a isotype but not of the other IgG subclasses (Fig. 7). This result, which is consistent with data reported by others with normal B cells (28, 29), was obtained in several independent experiments and was also observed with B lymphocytes from CD4-depleted mice infected with adenovirus (data not shown).

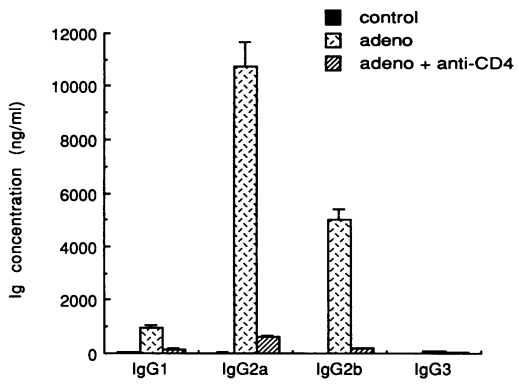

FIG. 3. Effect of CD4-positive cell suppression on IgG production after adenovirus (adeno) infection. IgGs were assayed in supernatants of spleen cell cultures initiated 10 days p.i. GK1.5 ascitic fluid was injected on days 0 and 5 p.i. Results are shown for the same groups presented in Table 2 (mean ± SE).

As viruses induce the secretion of IFN-γ by T lymphocytes (13), it was suspected that this cytokine mediated the IgG2a preponderance in antibodies produced during viral infections. Hence, we attempted to prevent the IgG2a production by injecting in vivo anti-IFN-γ MAbs, namely F3 (16), R4-6A2 (30), and XMG-1.2 (6), but without success (data not shown).

DISCUSSION

Adenovirus and LDV, while belonging to very different families, have the common property of inducing in vivo polyclonal activation of murine B lymphocytes. Using antibodies able to suppress T-helper-dependent immune functions, we have shown two different phases in this phenomenon: (i) a B-cell activation that was not affected by the injection of anti-T-helper-cell MAb and (ii) an IgG production, restricted to IgG2a and to a lesser extent to IgG2b, that could be suppressed by the same treatment. This observation suggests that T lymphocytes induce the secretion of IgG2a by B cells that are already activated. This hypothesis was supported by the inhibition of LDV-induced IgG secretion by a single injection of GK1.5 at 4 days p.i., when B lymphocytes were already proliferating (10).

TABLE 4. Effect of anti-CD4 MAb on immunoglobulin production after LDV infection[a]

LDV infection	Anti-CD4 treatment	Mean concn[b] (ng/ml) ± SE					
		IgM	IgG1	IgG2a	IgG2b	IgG3	IgA
−	−	934 ± 109	179 ± 44	61 ± 14	49 ± 16	10 ± 1	160 ± 34
+	−	1,324 ± 189	335 ± 53	2,499 ± 355	876 ± 243	15 ± 3	197 ± 62
+	+	1,266 ± 60	274 ± 23	96 ± 12	52 ± 2	10 ± 1	67 ± 15

[a] CBA/Ht mice were injected with 1 mg of GK1.5 MAb concomitantly with LDV infection. Immunoglobulins were assayed by ELISA in the supernatant of spleen cell cultures initiated 7 days p.i.

[b] Results are indicated for groups of four animals.

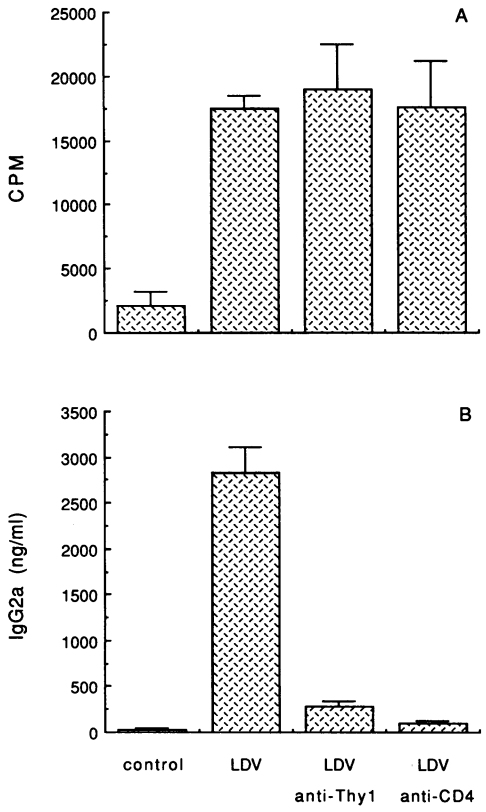

FIG. 4. Role of T lymphocytes in the B-lymphocyte polyclonal activation induced by LDV. CBA/Rij mice were infected with LDV and injected with anti-Thy1 (A6703A8) or with anti-CD4 (GK1.5) MAb on days −1, 2, and 5 p.i. (A) Thymidine incorporation by spleen cells was measured 1 week p.i. (mean ± SE for groups of four animals). (B) IgG2a was assayed by ELISA in the supernatant of spleen cell cultures initiated at the same time (mean ± SE).

The role of T-helper cells in virally induced B-lymphocyte proliferation could be similar in some aspects to that observed by others with a parasite such as *Trypanosoma cruzi*. In this case, anti-CD4 treatment abolished the polyclonal plaque-forming cell response induced by the infection (23). However, the suppression of T-helper functions depressed

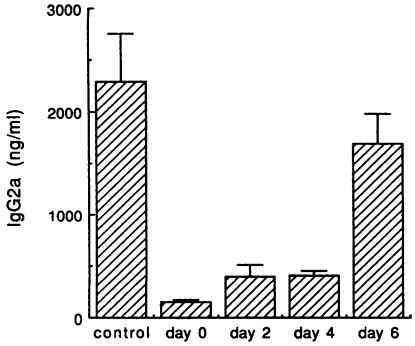

FIG. 5. Kinetics of anti-CD4 MAb injection after LDV infection. IgG2a levels were measured in supernatants of spleen cell cultures initiated 7 days after infection of CBA/Ht mice with LDV. The day p.i. on which GK1.5 ascitic fluid was administered is indicated, and results are shown for groups of four animals (mean ± SE).

TABLE 5. IgG subclass production after LPS stimulation of spleen cells from control mice and anti-CD4-treated LDV-infected animals[a]

LDV infection + anti-CD4 treatment	LPS	Mean IgG production (ng/ml) ± SE			
		IgG1	IgG2a	IgG2b	IgG3
−	−	4 ± 1	<3[b]	21 ± 12	26 ± 3
−	+	55 ± 7	11 ± 1	148 ± 22	287 ± 27
+	−	<3[b]	18 ± 5	17 ± 2	22 ± 7
+	+	26 ± 7	78 ± 16	95 ± 13	249 ± 10

[a] Spleen cells from control CBA/Ht mice or from animals infected with LDV and treated with anti-CD4 MAb 5 days before were cultured for 5 days in the presence or absence of LPS. Results of IgG assays in cell supernatants are expressed for groups of four mice.
[b] Limit of detection.

the B-cell blastogenic response triggered by the parasite (22, 23), in contrast to what we observed after viral infection.

How viruses can activate B lymphocytes is not fully understood. Some viral antigens, such as glycoproteins of Sendai and Sindbis viruses and adenovirus fiber protein, can induce a strong in vitro proliferative response of murine B cells which is in most cases T-cell independent (14, 15, 19). Influenza virus hemagglutinin displays a similar effect by its binding to the major histocompatibility complex present on murine B lymphocytes (2, 26, 27). Direct binding of LDV on the Ia molecule, suspected to be the viral receptor (17, 18), could similarly trigger the B-cell proliferation. However, the role of soluble factors, such as IL-6, a B-cell activator (31), whose secretion is induced by viruses (4), cannot be excluded.

Whereas virally induced proliferation of B lymphocytes seems to be largely T-cell independent, the role of T-helper cells in the secretion of immunoglobulins by such activated B cells was demonstrated by the effect of anti-CD4 antibody in infected mice. In addition, the selective production of various isotypes by B cells from T-depleted infected animals was restored by incubation with T-cell factors, such as IL-4 and IFN-γ for IgG1 and IgG2a, respectively. These obser-

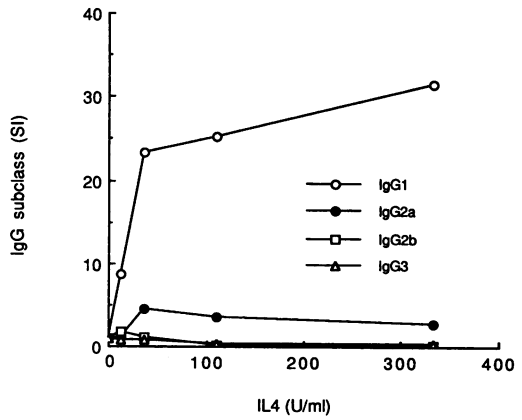

FIG. 6. Effect of IL-4 on IgG subclass production by B lymphocytes from CD4-depleted LDV-infected mice. IgGs were assayed in supernatants of B lymphocytes derived from CBA/Ht mice 5 days after infection with LDV and treatment with anti-CD4 MAb. Pooled cells from five animals were cultured for 6 days in the presence of LPS (20 μg/ml) and of serial doses of murine rIL-4. Stimulation index (SI) is the ratio between immunoglobulin levels in the supernatants of cells cultured with and without IL-4.

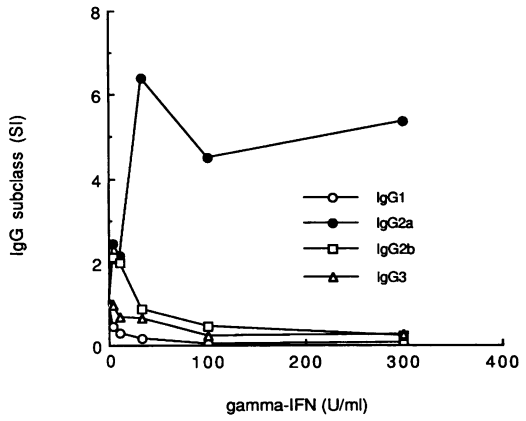

FIG. 7. Effect of IFN-γ on IgG subclass production by B lymphocytes from CD4-depleted LDV-infected mice. IgGs were assayed in supernatants of B lymphocytes derived from CBA/Ht mice 5 days after infection with LDV and treatment with anti-CD4 MAb. Pooled cells from five animals were incubated for 36 h in the presence of LPS (20 μg/ml) and of serial doses of murine rIFN-γ, washed, and cultured for 6 days in the presence of LPS alone. Stimulation index (SI) is the ratio between immunoglobulin levels in the supernatants of cells cultured with and without IFN-γ.

vations suggest that viruses do not suppress B-lymphocyte ability to produce other isotypes than IgG2a and that their influence on immunoglobulin switch could be mediated by the stimulation of a T-helper subtype secreting the appropriate lymphokines. IFN-γ, which is secreted by the Th1 subpopulation (6) and known to induce in vivo IgG2a responses (12), could quite possibly be involved in this phenomenon. However, we failed to inhibit IgG2a production by in vivo injection of anti-IFN-γ MAbs, either because they did not completely neutralize IFN-γ in virally infected mice or because alternative mechanisms were involved. Moreover, if Th1 lymphocytes were indeed responsible for this polyclonal IgG2a production, it would still have to be determined whether the viral infection influences the subtype of all T-helper cells, irrespective of their antigenic specificity, or only of virus-specific T-helper lymphocytes, which could in turn modify the isotype of IgG concomitantly secreted by all B cells.

The production of IgG2a as antiviral antibodies (9) could be a defensive advantage for the infected host, but is the polyclonal production of IgG2a of any help? Together with a polyclonal cytotoxic T-lymphocyte stimulation (33), the secretion of antibodies able to bind various antigens (J.-P. Coutelier, J. T. M. van der Logt, and F. W. A. Heessen, J. Autoimmun., in press), even with a low affinity, could represent a first defense, before more specific responses can be elaborated. However, without control, this general activation of the immune system could lead to disease (20). Therefore, identification of the mechanisms that induce and regulate this activation is of major interest.

ACKNOWLEDGMENTS

We are indebted to M. D. Gonzalez for expert technical assistance and to J. Van Snick, P. L. Masson, C. J. Pfau, and C. Godfraind for critical reading of the manuscript. F. W. Fitch, H. R. MacDonald, T. Mosmann, R. Devos, W. Müller, W. Fiers, and J. Van Snick are gratefully acknowledged for gifts of reagents.

This work was supported by the Fonds National de la Recherche Scientifique (FNRS), Fonds de la Recherche Scientifique Médicale (FRSM), Loterie Nationale, and the State Prime Minister's Office—Science Policy Programming (interuniversity attraction poles, grant 7, and concerted actions, grant 88/93-122), Belgium. J.-P. Coutelier is a research associate with the FNRS.

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