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Activated B cells can deliver help for the *in vitro* generation of antiviral cytotoxic T cells

(influenza viruses/cell-mediated immunity/immune regulation/helper T cells/lipopolysaccharide)

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ABSTRACT **The experiments described in this paper show that activated B cells can deliver help for antiviral cytotoxic T (T_c) cell responses *in vitro*. This conclusion is based on four observations. (*i*) Influenza viruses induced secondary T_c cell responses *in vitro* in the absence of $CD4^+$ T cells. This capacity correlated with the B-cell mitogenicity of these viruses. (*ii*) Depletion of both $CD4^+$ T cells and B cells prevented the generation of anti-influenza T_c cell responses, whereas depletion of either $CD4^+$ T cells or B cells alone failed to do so. In addition, supplementation of unprimed B cells restored the T_c cell responsiveness of primed splenocytes that had been depleted of both $CD4^+$ T cells and B cells. (*iii*) Contact between T and B cells was not obligatory for the delivery of B-cell helper signal, and hence help was mediated by a soluble factor(s). (*iv*) Lipopolysaccharide-activated B cells could replace the $CD4^+$ T-cell requirement in the induction of T_c cell responses to nonmitogenic influenza virus *in vitro*.**

The requirement for cell–cell collaboration in the generation of an immune response has been well established (1–3). $CD4^+$, class II major histocompatibility complex (MHC)-restricted T cells have been shown to have the capacity to provide help for both antibody responses and cytotoxic T (T_c)-cell responses (4, 5). The requirement for $CD4^+$ helper T (T_h) cells in antibody responses to most antigens is well documented (1, 6–8). In contrast, the requirement for $CD4^+$ T cells in the generation of class I MHC-restricted, antigen-specific T_c-cell responses remains controversial. *In vitro* such a requirement has been deduced from a variety of experimental approaches such as limiting-dilution analysis (9, 10), antibody blocking (11), cell depletion (12–14), and genetic manipulations (15, 16). However, $CD4^+$ T cells are not required in *in vivo* T_c-cell responses against a variety of viruses such as ectromelia virus (7), herpes simplex virus (17), lymphocytic choriomeningitis virus (8), vaccinia virus (unpublished data), and influenza virus (unpublished data). In these situations the helper cells for T_c-cell responses have not been identified.

The possibility that B cells may act as helper cells for T_c-cell responses has not been studied. Available data indicate that B cells may play an active role in T cell-mediated immune responses. Thus, activated B cells are efficient antigen-presenting cells for both class I and class II MHC-restricted, antigen-specific T-cell responses (18, 19). Mathematical modeling (20) of Lanzavecchia's data on B cell-mediated antigen presentation (21) indicates that B cells deliver two signals to T cells during antigen presentation, only one signal being antigen specific. In this context, activated B cells have been shown to produce lymphokines such as interleukin 2 (IL-2) (22, 23). In addition, B cells have been shown to influence the generation of the T-cell repertoire early in life (24). Furthermore, depletion of B cells *in vivo* affects a number of T-cell functions, such as the priming of T_h cells in lymph nodes (25–27), the development of experimental allergic encephalitis (28), and the kinetics of antiviral T_c cell responses (29).

We report here that type A influenza viruses, which are B-cell mitogens, can induce the generation of secondary anti-influenza virus T_c cell responses in the absence of $CD4^+$ T cells and demonstrate that B cells provide the necessary help in this system.

MATERIALS AND METHODS

Mice. Mice were bred at the John Curtin School of Medical Research. Female mice 6 to 8 weeks old were used.

Viruses. Influenza A virus strains A/WSN/33(H1N1) (A/WSN), A/Japan/305/57(H2N2) (A/Japan), A/Port Chalmers/1/73(H3N2) (A/PC) were grown and titrated as described (30).

Recombinant vaccinia virus, VV-PR8-NP6 (VV-NP) which expresses the nucleoprotein from influenza virus strain A/PR/8/34(H1N1) (31), was provided by D. Boyle (Australian Animal Health Laboratories, Geelong, Australia).

Generation of Secondary T_c-Cell Responses *in Vitro*. Splenocytes from mice primed intravenously (i.v.) with 10^3 hemagglutination units of A/WSN virus 7–14 days previously were stimulated *in vitro* with influenza virus A/WSN, A/Japan, or A/PC (10^{-4} hemagglutination units per splenocyte) or with VV-NP (20 plaque-forming units per splenocyte) as has been described (30). The method for the T_c-cell assay has been described in full (30, 32).

Depletion of Lymphocyte Subpopulations. Ig^+ B cells were depleted by rosetting of splenocytes with sheep anti-mouse Ig-coupled sheep red blood cells as has been described (32). $Thy\text{-}1^+$, $CD8^+$, and $CD4^+$ cells were depleted by repeated treatment with monoclonal antibodies (mAbs) plus complement as has been described (33). The mAbs used were: Anti-mouse Thy-1.2 (F7D5 clone, Bicester, Oxon, U.K.), rat anti-mouse CD4 (34), and rat anti-mouse CD8 (originally derived from R. W. Fitch, University of Chicago). Depletion of B and T cells was analyzed by fluorescence-activated cell sorting as has been described (35).

Marbrook Double Chamber Experiment. The two chambers consisted of two glass tubes separated by a polycarbonate membrane (0.4 μm, Nucleopore, Peasanton, CA 94566). Inner tubes (15 × 65 mm) contained 5.6×10^6 A/WSN-primed CBA/H splenocytes depleted of both $CD4^+$ and Ig^+ cells (Ig^- $CD4^-$). Outer tubes (22 × 150 mm) contained 10^7 unprimed CBA/H mouse splenocytes depleted

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Abbreviations: LPS, lipopolysaccharide; mAb, monoclonal antibody; VV-NP, recombinant vaccinia virus VV-PR8-NP6 expressing nucleoprotein from influenza virus A/PR/8/34 (H1N1); IL-2, interleukin 2; MHC, major histocompatibility complex; T_c and T_h cells, cytotoxic and helper T cells, respectively; A/WSN, A/WSN/33; A/Japan, A/Japan/305/57; A/PC, A/Port Chalmers/1/73; HAU, hemagglutination units.

of Thy-1$^+$ T cells (Ig$^+$ Thy-1$^-$) or CBA/H splenocytes depleted of both Ig$^+$ and Thy$^+$ (Ig$^-$ Thy-1$^-$) cells. Cells in both chambers were stimulated with 500 hemagglutination units of A/Japan virus in a 5-ml volume of Eagle's minimal essential medium. After 5 days of culture, the cells in the inner chambers were harvested, divided into two aliquots, and treated with complement either alone or with anti-mouse CD8 mAb as described above. T_c activity of the recovered cells was tested in an 8-hr assay with both A/WSN-infected and uninfected L929 cells as targets.

RESULTS

Efficacy of Cell Depletion. The efficacy of CD4$^+$ T-cell depletion is given in Table 1. In the two experiments shown, two successive treatments with mAb plus C complement reduced the frequency of CD4$^+$ T cells from around 13% to undetectable levels. CD4$^+$ T cells remained undetectable after 5 days of *in vitro* culture, which increased the frequency of CD4$^+$ T cells in undepleted splenocyte population. The successful depletion of Ig$^+$ B cells and Thy-1$^+$ T cells was confirmed by fluorescence-activated cell sorter flow cytometry and the abrogation of proliferative responses to B- and T-cell mitogens, lipopolysaccharide (LPS), and Con A, respectively.

Correlation Between Mitogenicity of Influenza Viruses and Their Capacity to Induce T_c-Cell Response in Absence of CD4$^+$ T Cells. In preliminary experiments, we found that influenza A virus could induce a potent antiviral T_c-cell response in the absence of CD4$^+$ T cells and decided to study this phenomenon further.

Influenza viruses are T cell-independent B-cell mitogens (36), and mitogenicity varies according to the subtypes of hemagglutinin, H2 (A/Japan) virus being the most potent mitogen, whereas H1 (A/WSN) and H3 (A/PC) are only moderately mitogenic (36–38). Furthermore, optimal expression of mitogenicity of the H2-type virus depends on class II I-E antigen expression on the B cell (39, 40). To test if the B-cell mitogenicity of influenza virus was responsible for the lack of CD4$^+$ T-cell requirement for anti-influenza T_c cell responses *in vitro*, four strains of inbred mice differing in their I-E expression, CBA/H (*H-2^k*, I-E$^+$), BALB/c (*H-2^d*, I-E$^+$), BALB.B (*H-2^b*, I-E$^-$), and C57BL/6J (*H-2^b*, I-E$^-$) were primed with A/WSN, and their splenocytes were depleted of CD4$^+$ T cells or left undepleted and stimulated *in vitro* with A/Japan virus. A/Japan virus induced a significant proliferative response in B cells from BALB/c and CBA/H mice, which express I-E antigen (data not shown). Depletion of CD4$^+$ T cells had little effect in the T_c-cell responses generated in these two mouse strains (Fig. 1). However, the same virus induced at best marginal proliferation in B cells from BALB.B and C57BL/6J mice, which do not express I-E antigen, and depletion of CD4$^+$ T cells significantly reduced the anti-influenza T_c-cell responses in splenocytes from these two mouse strains (Fig. 1). In addition, influenza virus-primed splenocytes from CBA/H mice depleted of CD4$^+$ T cells were tested for their ability to generate T_c cells in response to viruses with varying B-cell mitogenicity (38, 39). Depletion of CD4$^+$ T cells prevented the generation of anti-influenza virus T_c-cell responses induced by VV-NP, which is nonmitogenic to B cells, whereas the generation of T_c-cell responses induced by A/Japan virus was hardly affected. Depletion of CD4$^+$ T cells significantly reduced T_c-cell responses when moderate B-cell mitogens, A/WSN and A/PC, were used as stimulator antigens (Fig. 2). The three strains of influenza viruses used here were tested for their mitogenicity on purified Ig$^+$ splenocytes. The strength of B-cell proliferation correlated with their lack of CD4$^+$ T-cell requirement (data not shown).

Table 1. Frequencies of CD4$^+$ T cells in CBA/H spleen cell populations as determined by flow cytometry

Treatments	CD4$^+$ T cells, %			
	Prior to culture		After culture	
C	12.4	13.1	25.4	24.9
Anti-CD4 + C	0.5	0.0	1.0	0.0

*Splenocytes from A/WSN-primed mice were treated with anti-CD4 mAb plus complement (C) or complement alone and were cultured for 5 days *in vitro*. Two independent experiments are shown. The frequencies of CD4$^+$ T cells were determined by single-color flow cytometry.

Requirement for B Cells in the Generation of Secondary T_c-Cell Responses in the Absence of CD4$^+$ T Cells. *Negative selection experiments.* Splenocytes from A/WSN-primed CBA/H mice were either depleted of CD4$^+$ T cells and Ig$^+$ B cells (CD4$^-$ Ig$^-$), Ig$^+$ B cells alone (Ig$^-$), or CD4$^+$ T cells alone (CD4$^-$). These responder cells were stimulated with A/Japan virus for 5 days. Ig$^-$ and CD4$^-$ splenocytes generated a reduced but still highly significant anti-influenza virus T_c-cell response. However, CD4$^-$ Ig$^-$ splenocytes failed to do so (Fig. 3).

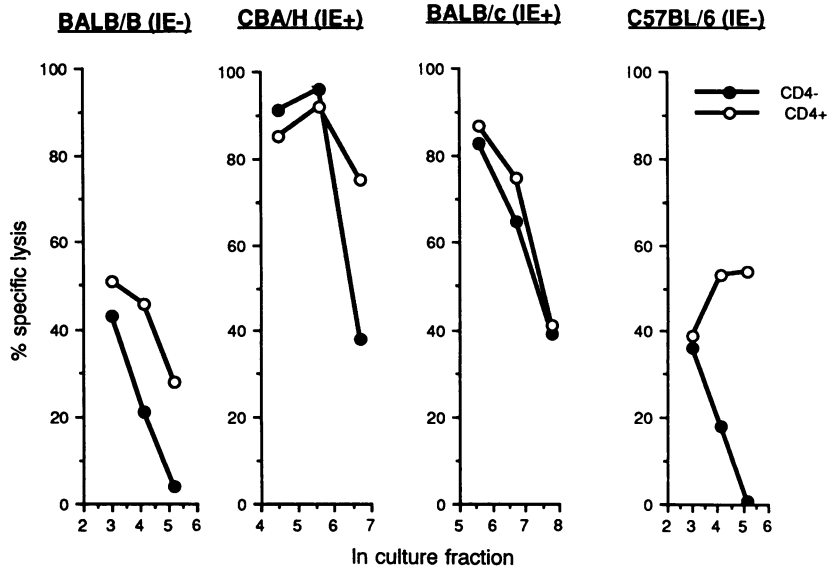

FIG. 1. The effect of CD4$^+$ T-cell depletion on the T_c-cell responses induced by A/Japan virus *in vitro*. Aliquots of 5 × 10^7 A/WSN-primed splenocytes from four mouse strains differing in their H-2 and I-E antigen expression were depleted of CD4$^+$ cells (CD4$^-$) or left undepleted (CD4$^+$) and stimulated with A/Japan virus in 40 ml of medium. T_c-cell activity was determined in an 8-hr assay using uninfected and A/WSN-infected targets. The targets used were: L929 (H-2^k, for CBA/H effectors), P815 (H-2^d, for BALB/c effectors), and EL-4 (H-2^b, for BALB.B and C57BL/6J effectors). The data shown are percentages of specific lysis on infected targets with that of the uninfected targets subtracted. All points shown are means of triplicate results with SEM never greater than 5%.

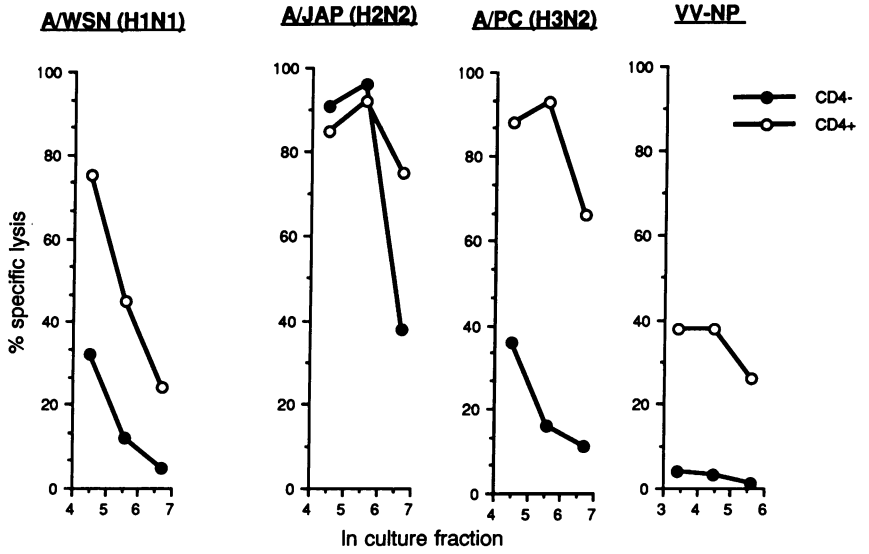

FIG. 2. The effect of CD4$^+$ T-cell depletion on the *in vitro* T_c-cell responses induced by A/WSN, A/Japan, A/PC, and VV-NP, respectively. Aliquots of 5×10^7 splenocytes from A/WSN-primed CBA/H mice depleted of CD4$^+$ cells (CD4$^-$) or left undepleted (CD4$^+$) were stimulated with viruses. See the legend to Fig. 1.

Positive selection experiment. A/WSN-primed CBA/H Ig$^-$ CD4$^-$ splenocytes were supplemented with unprimed syngeneic splenocytes depleted of either T cells (Ig$^+$ Thy-1$^-$) or B cells and T cells (Ig$^-$ Thy-1$^-$). These responder populations were stimulated with A/Japan virus for 5 days. Primed Ig$^-$ CD4$^-$ splenocytes, when supplemented with unprimed Ig$^+$ Thy-1$^-$ splenocytes, generated a potent T_c-cell response, while the same splenocytes supplemented with unprimed Ig$^-$ Thy-1$^-$ splenocytes gave no detectable T_c-cell response, as did those cultures that received no cells (Fig. 4). Thus B cells can substitute for CD4$^+$ T_h cells in the generation of anti-influenza virus T_c-cell responses.

The "Help" Provided by B Cells Is Mediated by Soluble Factor(s). To investigate whether contact between T and B cells is obligatory for the generation of T_c-cell responses, a Marbrook-type chamber was constructed. The T_c-cell response in the inner chamber (Ig$^-$ CD4$^-$) was 5-fold higher when unprimed Ig$^+$ Thy-1$^-$ splenocytes instead of an equal number of Ig$^-$ Thy-1$^-$ splenocytes were in the outer chamber (Fig. 5). Treatment with anti-murine CD8 mAb plus complement abolished T_c cell activity; hence, the cells that caused target-cell lysis are conventional CD8$^+$ T_c cells.

LPS-Activated B Cells Can Provide the Necessary Help for T_c-Cell Responses. To test whether B cells activated by mitogens other than influenza virus can also provide help for T_c-cell responses, a low concentration of LPS (1 μg/ml) was used to activate B cells. VV-NP recombinant virus is nonmitogenic to lymphocytes (data not shown) and could not induce *in vitro* secondary T_c-cell responses in the absence of CD4$^+$ T cells (Fig. 2). However, supplementation of A/WSN-primed Ig$^-$ CD4$^-$ splenocytes with B cells and LPS, but not with LPS alone, restored T_c-cell responses (Fig. 6). In one experiment (Fig. 6 *Left*) the number of A/WSN-primed Ig$^-$ CD4$^-$ splenocytes was kept constant, whereas in the other experiment (Fig. 6 *Right*) the total cell number was kept constant. In both experiments, T_c-cell responses were generated if and only if both B cells and LPS were present. Lysis of uninfected target cells was high in both experiments, characteristic of VV-NP-induced anti-influenza virus T_c-cell responses *in vitro* (data not shown).

DISCUSSION

We report here that activated B cells can deliver help for the generation of anti-viral T_c-cell responses *in vitro*. This conclusion is based on four observations.

(*i*) Influenza virus, as an exception to the general rule (11–14), can induce class I MHC-restricted antigen-specific T_c-cell response in the absence of CD4$^+$ T_h cells *in vitro*. Data reported here show a correlation between the capacity of influenza viruses to induce T_c-cell responses in the absence of CD4$^+$ T cells and their B cell mitogenicity. Thus, A/Japan virus, which is a potent mitogen (36, 37) for B cells, induced T_c-cell responses in the absence of CD4$^+$ T cells comparable to that in the presence of CD4$^+$ T cells; A/WSN and A/PC,

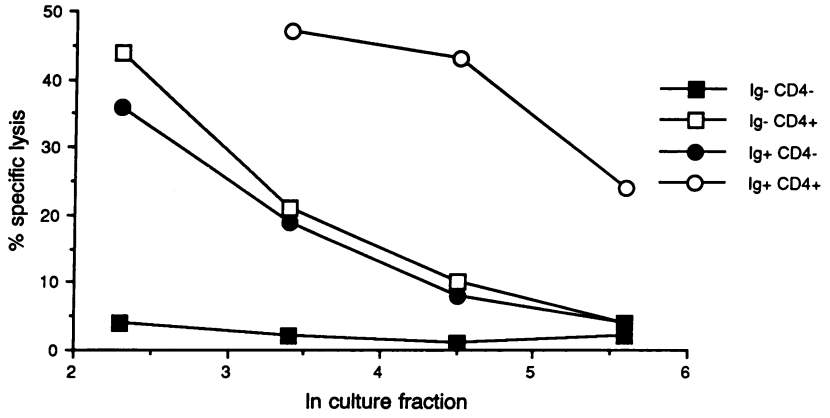

FIG. 3. The effect of CD4$^+$ T- and B-cell depletion of T_c-cell responses induced by A/Japan virus *in vitro*. A/WSN-primed CBA/H splenocytes were left undepleted (CD4$^+$ Ig$^+$) or depleted of CD4$^+$ T cells (Ig$^+$ CD4$^-$), B cells (Ig$^-$ CD4$^+$) or depleted of CD4$^+$ T cells and B cells (CD4$^-$ Ig$^-$) and used as responder cells. Responder cells (5×10^6) were stimulated with A/Japan virus in 5 ml of medium, and T_c-cell activity was tested. The data shown are percentages of specific lysis on A/WSN-infected L929 cells with that on uninfected targets subtracted. All points shown are means of triplicates, with the SEM all below 2.2%.

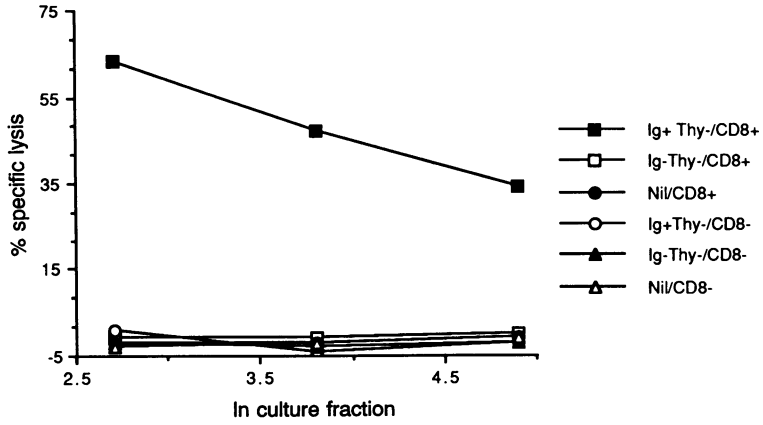

FIG. 4. Restoration of T_c-cell responses induced by A/Japan virus *in vitro* by cell supplementation. A/WSN-primed splenocytes were depleted of $CD4^+$ T and Ig^+ cells (Ig^- $CD4^-$), and 3×10^6 Ig^- $CD4^-$ splenocytes were supplemented with medium alone (Nil), 3×10^6 unprimed splenocytes depleted of Thy-1^+ T cells (Ig^+ Thy-1^-) or T and B cells (Ig^- Thy-1^-). These responder cells were stimulated with A/Japan virus in 5 ml of medium. The effector cells were depleted of $CD8^+$ cells ($CD8^-$) or left undepleted ($CD8^+$), and their T_c-cell activity was determined. See the legend to Fig. 3.

which are moderate B-cell mitogens (36, 37), induced lower but significant T_c-cell responses in the absence of $CD4^+$ T cells; VV-NP, which is nonmitogenic to B cells, failed to induce a detectable T_c-cell response in the absence of $CD4^+$ T cells. A similar correlation was found when A/Japan virus was used to stimulate T_c-cell responses in splenocytes from mice that express or do not express H-2I-E antigen. The optimal expression of the mitogenicity of A/Japan viruses depends on the I-E antigen expression on B cells (39, 40). A/Japan virus is highly mitogenic to B cells from BALB/c and CBA/H mice expressing I-E antigens but is only marginally mitogenic to B cells from BALB.B and C57BL/6J mice lacking I-E antigens. Depletion of $CD4^+$ T cells from primed splenocytes significantly reduced T_c-cell responses in splenocytes from BALB.B and C57BL/6J mice but hardly in splenocytes from BALB/c and CBA/H mice.

(*ii*) The induction of $CD4^+$ T_h-independent T_c-cell responses depended on the presence of B cells. Depletion of both $CD4^+$ T cells and B cells prevents the generation of T_c-cell responses. However, significant, although reduced, T_c-cell responses can be generated in the absence of either $CD4^+$ T_c cells or B cells. Supplementation of B cells restored T_c-cell responses in $CD4^+$ T and B cell-depleted cultures. These results indicate that $CD4^+$ T cells and B cells can provide help for T_c-cell responses in this system.

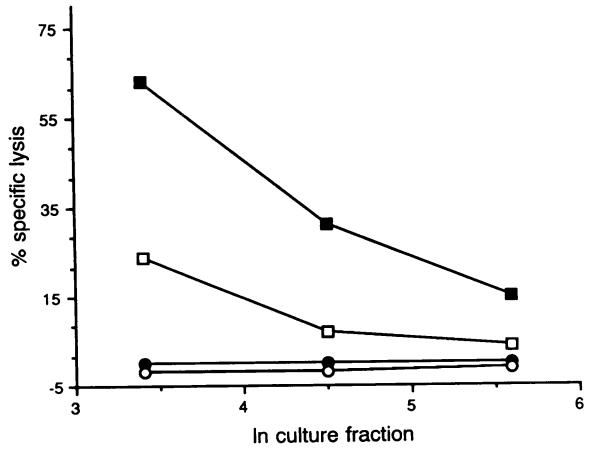

FIG. 5. T_c-cell responses generated in Marbrook double-chamber cultures. The A/WSN-primed Ig^- $CD4^-$ CBA/H splenocytes were put in the inner chamber. Either Ig^+ Thy-1^- (solid symbols) or Ig^- Thy-1^- (open symbols) unprimed CBA/H splenocytes were put in the outer chamber. The effectors generated in the inner chamber were depleted of $CD8^+$ (circles) cells or were left undepleted (squares), and the T_c-cell activity was determined on A/WSN-infected and uninfected L929 cells. The specific lysis (not shown) on uninfected L929 cells was always less than 0.1%. All points shown were means of triplicates, with the SEM never greater than 2.2%.

(*iii*) Direct T cell–B cell contact is not obligatory for the generation of $CD4^+$ T cell-independent T_c-cell responses, as T and B cells separated by a 0.4-μm-pore-size membrane can collaborate successfully. This result excludes the possibility that activated B cells are superior antigen-presenting cells and obviate the $CD4^+$ T-cell requirement in this way. This result also indicates that B cells secrete one or more soluble factors that can induce and/or potentiate T_c-cell responses. The nature of this soluble factor(s) is at present unknown. However, preliminary data indicate that the factor(s) involved is not interleukin 2 (IL-2) or IL-4, as the supernatant from activated B cells that can provide the necessary help does not contain IL-2 and IL-4 activity (data not shown). It is known that certain $CD8^+$ T cells can secrete IL-2 (41) and B cells can produce IL-1 (42). A recent study suggests that IL-1 may potentiate the production of IL-2 by $CD8^+$ T cells (43). This raises the possibility that B cells may provide IL-1,

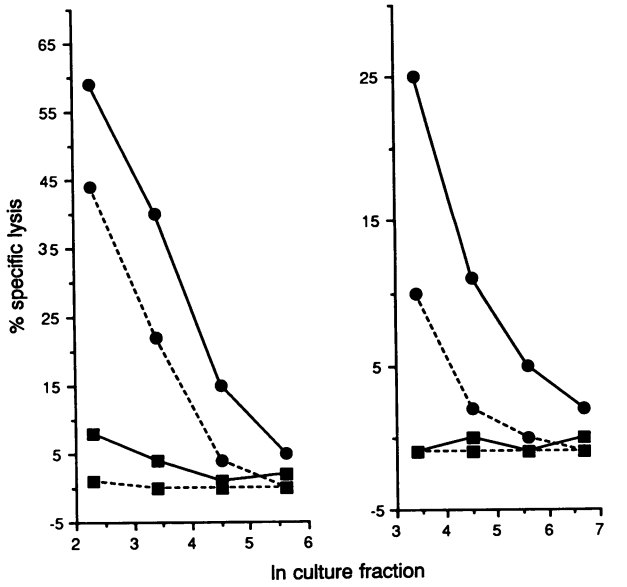

FIG. 6. VV-NP-induced secondary anti-influenza virus T_c-cell response in the presence of LPS *in vitro*. A/WSN-primed Ig^- $CD4^-$ CBA/H splenocytes, supplemented with syngeneic, unprimed Ig^+ Thy-1^- splenocytes (●) or medium only (■), were stimulated with VV-NP in the presence of LPS. The T_c-cell activity generated was tested on uninfected (broken lines) and A/WSN-infected (unbroken lines) L929 cells. (*Left*) Primed Ig^- $CD4^-$ splenocytes (5×10^6) were supplemented with 3×10^6 Ig^+ Thy-1^- unprimed splenocytes or medium only and were cultured in 5 ml of medium. (*Right*) Either 2.0×10^7 primed Ig^- $CD4^-$ splenocytes or 1.3×10^7 Ig^- $CD4^-$ primed plus 7×10^6 unprimed Ig^+ Thy-1^- splenocytes were cultured in 20 ml of medium. The data shown were means of triplicates, with all SEM below 1%.

which induces IL-2 production by $CD8^+$ T cells, and IL-2 provides the help for T_c-cell responses. Our experiments described here indicate that this is unlikely to be the case. LPS, a classic inducer of IL-1 production by macrophages (44), failed to potentiate anti-influenza T_c-cell responses in primed $CD4^-$ Ig^- splenocytes (Fig. 6), which contain macrophages (as determined by nonspecific esterase staining; data not shown) as well as $CD8^+$ T cells.

(*iv*) The requirement for $CD4^+$ T cells in T_c-cell responses induced by nonmitogenic viruses can be obviated by the addition of a low concentration of LPS, a conventional B-cell mitogen.

It is surprising that B-cell helper functions for T cells have not been described previously to our knowledge. Helper cells for anti-influenza virus T_c-cell responses have been described as typically Thy-1^+ Ig^- Lyt-1^+ T cells (9). A possible explanation may be the use of irradiated mouse splenocytes as helper populations. Irradiation might have prevented B-cell activation. The general difficulty in showing B-cell helper function for T_c-cell generation may be the $CD4^+$ T_h^- dependency of B-cell activation (45). However, because B-cell activation *in vivo* is a consequence of most infections or immunizations, it is conceivable that B cells could be required for optimal T-cell responses. The obligatory role of antigen-specific B cells for T-cell priming in lymph nodes (25–27), the requirement of carrier-specific B cells for optimal generation of hapten-specific antibody-producing cells (46, 47), and the failure to induce experimental allergic encephalitis in B cell-deficient rats (28) are observations consistent with a B-cell helper function. It is interesting to note that depletion of B cells resulted in a significant reduction of massive lymph node T-cell proliferation in MRL-*lpr*/*lpr* mice *in vivo* (25). Furthermore, recent data demonstrates an ability of splenic B cells and B-cell lines to release lymphokines such as IL-2 (22, 23). Whether activation of helper B cells is the explanation for the recent findings that anti-viral T_c cells can be generated *in vivo* in the absence of $CD4^+$ T cells (7, 8, 17) should be tested.

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