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Immunology

T helper cell subsets require the expression of distinct costimulatory signals by antigen-presenting cells

(T-cell clones/macrophage/B cell/interleukin 1/interferon γ)

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ABSTRACT **We examined the ability of macrophages and B cells to function as antigen-presenting cells (APCs) for murine T_H1 and T_H2 cloned T helper cell lines. Antigen presented by concanavalin A-elicited peritoneal macrophages or resting splenic B cells stimulated antigen-dependent proliferation of both T helper subsets. Paraformaldehyde fixation of the APCs following different conditions of activation indicated differential requirements for costimulatory signals by T_H1 and T_H2 cells. T_H2 proliferative responses were strictly dependent on APC expression of IL-1. T_H1 proliferation was dependent on APC expression of a non-IL-1 costimulatory signal present on freshly isolated macrophages and on splenic B cells activated with anti-immunoglobulin plus interferon γ.**

T lymphocytes have traditionally been divided into two populations on the basis of reciprocal expression of CD4 and CD8 surface glycoproteins (1). More recently, two sets of murine $CD4^+$ T helper (T_H) cell clones were defined based on distinct patterns of lymphokine production (2, 3). T_H1 clones produce interferon γ (IFN-γ) and interleukin 2 (IL-2) but not interleukin 4 (IL-4) when activated by specific antigen or mitogens, utilize IL-2 as an autocrine growth factor, and mediate a delayed hypersensitivity response on passive transfer. T_H2 clones produce IL-4 but not IL-2 or IFN-γ, utilize IL-4 for autocrine growth, and fail to induce delayed responses. Their roles in providing B-cell help are more controversial, but there appear to be important differences regarding immunoglobulin isotype regulation (4, 5). The distinct properties of these two subtypes suggest that they are likely to have different roles in regulating the immune response. It is therefore of importance to gain an understanding of the mechanisms by which the two populations are derived, activated, or selected during an antigenic challenge. Since both subtypes recognize foreign antigen in association with class II major histocompatibility complex (MHC)-encoded molecules, it is reasonable to assume that class II-bearing antigen-presenting cells (APCs) such as macrophages and B cells may play a role in modulating T_H1 and T_H2 responses. The aim of the present study was to determine whether the two types of T_H clones differ regarding requirements for these two APC types.

MATERIALS AND METHODS

Mice. Female CBA/J, BALB/cJ, and C57BL/6J and male B10.A/SgSnJ mice, purchased from The Jackson Laboratory, were used at 6–10 weeks of age.

Cell Lines. The generation and maintenance of the cloned T_H cell lines D1.5 (6), 11/3B (7), A.E7 (8), D10.G4.1 (D10) (9), and C4 (10) have been described. We thank Abul Abbas, Giampietro Corradin, Ronald Schwartz, and Charles Janeway, Jr., for supplying some of these clones. The clones CIB4 and CIG12 were generated and maintained in our laboratory according to the protocol described for C4 with the exception that these clones were derived from lymph node and spleen, respectively, of CBA/J mice immunized with the hen egg-white lysozyme-(46–61)-peptide [HEL-(46–61)] (11) rather than the entire HEL molecule. The G2 clone was similarly generated from B10.A mice immunized with pigeon cytochrome *c*. Clone 37 was provided by Andrew Glasebrook (Lilly Research Laboratories, La Jolla, CA).

Antigens, Lymphokines, and Antibodies. Rabbit gamma globulin, pigeon cytochrome *c*, ovalbumin, conalbumin, HEL, and concanavalin A (Con A) were purchased from Sigma. Bacterial lipopolysaccharide (LPS) was purchased from Ribi Research (Hamilton, MT). The synthetic peptide $(Asn\text{-}Ala\text{-}Asn\text{-}Pro)_3\text{-}Asn\text{-}Ala$ [$(NANP)_3NA$], representing the repeating immunodominant epitope of the *Plasmodium falciparum* circumsporozoite protein, was provided by Giampietro Corradin (7). The peptide HEL-(46–61) was provided by Paul Allen (11). The pigeon cytochrome *c*-(94–103)-peptide (12) and the ovalbumin-(323–339)-peptide (13) were synthesized on a RaMPS apparatus (DuPont) and purified by reversed-phase HPLC. In some experiments, we used the pigeon cytochrome *c* cyanogen bromide fragment 81–104 (14), a gift of Marc Jenkins and Ronald Schwartz (National Institutes of Health).

Recombinant murine IFN-γ and tumor necrosis factor α (TNF-α) were provided by Genentech (South San Francisco, CA). Recombinant murine IL-4 was donated by Monsanto. Recombinant murine IL-2 was purchased from Genzyme (Boston). Recombinant murine IL-1α was that of Fuhlbrigge *et al.* (15).

A monoclonal antibody to murine IL-1α, prepared by protein A purification of culture supernatants from the hamster–mouse hybridoma ALF161.1 (15), was kindly provided by Robert Fuhlbrigge. The anti-IL-2 receptor antibodies 7D4 and PC61 were purified by ammonium sulfate precipitation (0–50% cut) or passage over a protein A-Sepharose column, respectively. Bet-2, an anti-mouse IgM antibody, was purchased from the American Type Culture Collection and purified by precipitation with ammonium sulfate at 50% saturation. The antibodies AT83A (anti-Thy-1.2) and GK1.5 (anti-L3T4) were provided by Frank Fitch (University of Chicago). The hybridoma 11B11 (anti-mouse IL-4) was provided by William Paul (National Institutes of Health).

Macrophage Preparation. Con A-elicited macrophages were harvested from the peritoneal cavity of mice injected 3 days previously with 60 μg of Con A in 1 ml of 0.9% NaCl. Cells were cultured in flat-bottomed 96-well plates (Costar

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Abbreviations: anti-Ig, anti-immunoglobulin; APC, antigen-presenting cell; Con A, concanavalin A; HEL, hen egg-white lysozyme; IFN-γ, interferon γ; IL-*n*, interleukin *n*; LPS, lipopolysaccharide; MHC, major histocompatibility complex; mIL-1, membrane IL-1; $(NANP)_3NA$, $(Asn\text{-}Ala\text{-}Asn\text{-}Pro)_3\text{-}Asn\text{-}Ala$; T_H, T helper; TNF, tumor necrosis factor.

3596) at a density of 2×10^5 cells per well in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 mg of penicillin and 100 mg of streptomycin per ml, and 50 μM 2-mercaptoethanol. After culture for 2 hr at 37°C, nonadherent cells were removed and 100 μl of fresh medium containing IFN-γ (20 ng/ml) was added to each well. Some wells also received LPS (1 μg/ml) and/or antigen at various times following the addition of IFN-γ. The cells were cultured an additional 14 hr and then fixed for 15 min at room temperature with 1% (wt/vol) paraformaldehyde in medium. The cells were washed extensively, incubated at 37°C for 6–24 hr, and washed again prior to use in assays. In those experiments using live macrophages, Con A-elicited cells were treated with γ-radiation (3000 rads; 1 rad = 0.01 Gy) before addition to culture wells.

B-Cell Preparation. Spleen-cell suspensions were depleted of T cells by incubation with a mixture of AT83A (anti-Thy-1.2) and GK1.5 (anti-L3T4) culture supernatants for 30 min on ice followed by treatment with 10% preabsorbed guinea pig complement (Colorado Serum, Denver) for 45 min at 37°C. Viable B cells were recovered from the 1.075/1.091-g/ml interface of a Percoll density gradient (Pharmacia) spun at $750 \times g$ for 20 min at room temperature. The recovered cells were washed and then cultured in plastic tissue culture dishes for 2 hr at 37°C to remove adherent cells. High-density, resting B cells used in some experiments were recovered from the 1.081/1.091 g/ml interface of a Percoll gradient. Purified B cells were stimulated for 48 hr at 2×10^6 cells per ml in medium containing 10% (vol/vol) anti-mouse IgM antibody (Bet-2) and various lymphokines. The cells were washed, fixed with 1% paraformaldehyde, washed again, and then incubated overnight at 37°C to remove residual paraformaldehyde.

Assays. Proliferative responses of T-cell clones were assessed by using cells isolated on a Ficoll-Paque gradient (Pharmacia) 12–15 days after restimulation with antigen and irradiated spleen cells. The cultures were incubated for 72 hr at 37°C, with [*methyl*-^{3}H]thymidine (0.4 μCi per well; 1 μCi = 37 kBq) present for the final 18 hr of culture. Radioactivity incorporated into DNA was measured by liquid scintillation counting. To determine the T_H subset of the clones, T-cell supernatants were generated and assayed for IL-2, IL-4, and IFN-γ content. Supernatants were collected at 24 hr for IL-2 and IL-4 assay and at 72 hr for IFN-γ assay. IL-2 or IL-4 content of the 24-hr supernatants was assessed by their ability to support the growth of the CTLL cell line in the absence or presence of antibodies to the IL-2 receptor (7D4 and PC61) or IL-4 (11B11). IFN-γ content in the 72-hr T-cell supernatants was quantitated in an ELISA using the H1.5 monoclonal antibody specific for IFN-γ (16).

RESULTS

The panel of CD4$^+$, CD8$^-$ T-cell clones used in this study are listed in Table 1. When stimulated, the clones D1.5, G2, 11/3B, and 37 produced IFN-γ and IL-2 but not IL-4. A.E7 also produced IFN-γ but relatively little detectable IL-2 and no IL-4. These are defined as T_H1 clones. The clones D10, C4, CIB4, and CIG12 had a reciprocal pattern of lymphokine production, each producing IL-4 but no detectable IL-2 or IFN-γ and are, thus, T_H2 clones.

Macrophage Antigen Presentation to T_H1 and T_H2 Clones. Initial experiments compared the APC requirements of T_H1 and T_H2 clones by using live, irradiated peritoneal macrophages elicited with Con A, expressing high levels of class II MHC molecules. Both T_H1 and T_H2 clones proliferated when cultured with the appropriate number of Con A-elicited macrophages and antigen (Fig. 1). Optimal responses were induced by 5×10^3 to 5×10^4 macrophages per well. Higher macrophage densities were uniformly inhibitory.

Additional experiments were performed with Con A-elicited macrophages fixed with paraformaldehyde after various conditions of activation *in vitro*. The use of fixed APCs allowed us to control the expression of costimulator activities such as membrane IL-1 (mIL-1) and thus to evaluate potential differences in T_H1 and T_H2 costimulator requirements not apparent from experiments using live APCs. Shown in Fig. 2 are the proliferative responses of T_H1 and T_H2 clones cocultured with Con A-elicited macrophages that were fixed after 24 hr of culture with IFN-γ so as to maintain their expression of class II MHC molecules. One set of macrophages did not express mIL-1 (unstimulated), whereas the second set was exposed to LPS prior to fixation so as to induce it. All of the T_H1 clones responded comparably to both fixed macrophage populations. The T_H1 responses were unaffected by addition of exogenous IL-1 or a monoclonal

Table 1. T-cell clones: Specificities and lymphokine production

Clone	Mouse strain	MHC restriction	Antigen (peptide) specificity	IFN-γ,* ng/ml	CTLL [^{3}H]thymidine incorporation,† cpm $\times 10^{-3}$		
					No antibody	Anti-IL-2R	Anti-IL-4
T_H1							
D1.5	BALB/c	I-A^d	Rabbit gamma globulin (ND‡)	112	26.3	*2.1*	29.2
37	BALB/c	I-A^d	Ovalbumin (323–339)	12	31.2	*7.4*	33.3
A.E7	B10.A	I-E^k	Cytochrome *c* (94–103)	29	2.8	*0.3*	2.6
G2	B10.A	I-E^k	Cytochrome *c* (94–103)	94	32.4	*5.2*	29.6
11/3B	C57BL/6	I-A^b	*P. falciparum* circumsporozoite protein [(NANP)$_3$NA]	79	26.7	*1.7*	28.4
T_H2							
D10	AKR/J	I-A^k	Conalbumin (ND‡)	–	17.7	15.1	*0.2*
C4	CBA/J	I-A^k	HEL (46–61)	–	15.9	14.6	*2.1*
CIB4	CBA/J	I-A^k	HEL (46–61)	–	16.4	15.6	*0.6*
CIG12	CBA/J	I-A^k	HEL (46–61)	–	14.9	16.7	*0.5*

*The IFN-γ content of supernatants from 72-hr stimulated T-cell cultures was determined by ELISA. Data are presented as means of triplicate determinations. SDs were <10% of the mean.

†IL-2 and IL-4 content in 24-hr culture supernatants of antigen-stimulated T cells was determined by stimulation of CTLL cells in the absence or presence of antibodies to the IL-2 receptor (IL-2R) or to IL-4. Supernatants were incubated at 10% (vol/vol) with 5×10^3 CTLL cells, and [^{3}H]thymidine incorporation for the final 24 hr of a 48-hr culture was measured. 7D4 and PC61 (anti-IL-2 receptor antibodies) were used as a mixture (each at 10 μg/ml) and 11B11 culture supernatants (anti-IL-4) were added to a final concentration of 25% (vol/vol). Data are presented as means of triplicate determinations. SDs were <15% of the mean. Values indicating significant inhibition are shown in italic. Control values were as follows: IL-2 (10 units/ml), 33.1 (cpm $\times 10^{-3}$); IL-2 plus anti-IL-2R, 0.4; IL-2 plus anti-IL-4, 32.1; IL-4 (50 units/ml), 16.8; IL-4 plus anti-IL-2R, 15.4; IL-4 plus anti-IL-4, 0.2.

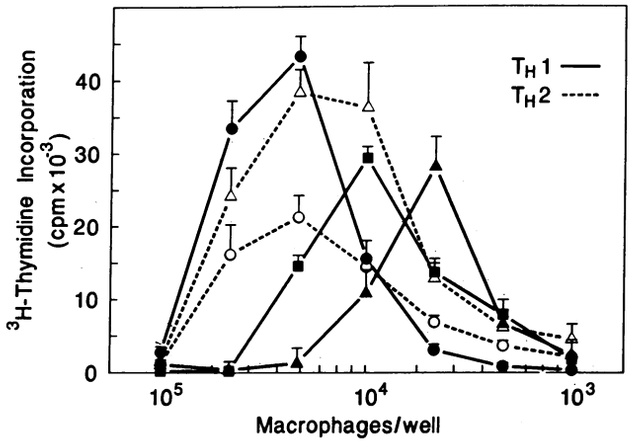

FIG. 1. Proliferative responses ([^{3}H]thymidine incorporation) of T_H1 and T_H2 clones to live macrophages. T cells (2×10^4) were cultured for 72 hr with the indicated number of irradiated, Con A-elicited macrophages of the appropriate MHC haplotype plus antigen. ●, A.E7 plus 10 μM cytochrome *c*; ■, 11/3B plus 100 nM $(NANP)_3NA$; ▲, D1.5 plus 1 μM rabbit gamma globulin; △, D10 plus 1 μM conalbumin; ○, CIB4 plus 10 μM HEL. Each value represents the mean of triplicate determinations.

antibody to IL-1α that completely inhibits mIL-1 (15). In striking contrast, the T_H2 clones responded only to fixed macrophages stimulated by LPS to express mIL-1. This response was completely inhibited by the anti-IL-1α antibody. T_H2 responded to unstimulated fixed macrophages when exogenous IL-1 was added.

Experiments in which macrophages were cultured for various times before fixation suggested that T_H1 clones required APC expression of an activity that was labile. LPS-stimulated macrophages fixed after brief culture *in vitro* (20 hr) were efficient APCs for both T_H1 and T_H2 clones (data not shown). However, after longer culture (96 hr), the responses of T_H1 clones were lost, whereas T_H2 responses to the same macrophage populations were maintained. A similar activity was found on freshly harvested macrophages activated *in vivo* with the intracellular pathogen *Listeria monocytogenes*, and it also decayed after *in vitro* culture.

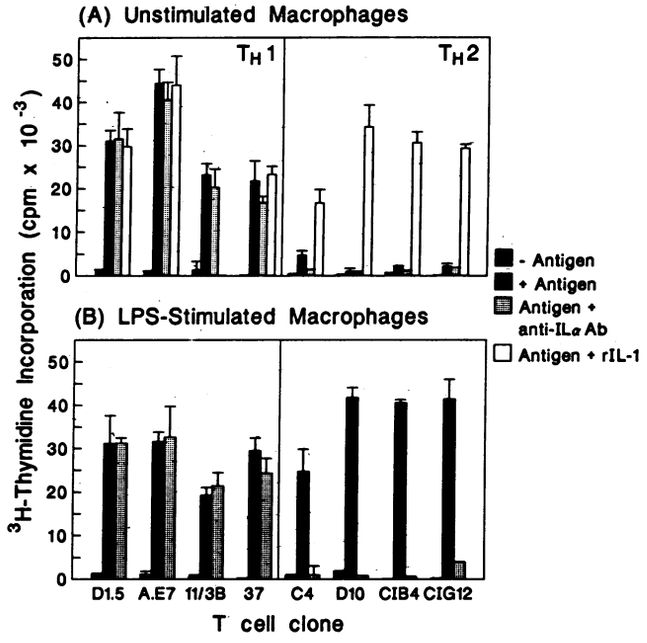

FIG. 2. Differential stimulation of T_H1 and T_H2 clones by fixed macrophages. Con A-elicited macrophages (2×10^5) from CBA/J, BALB/c or C57BL/6 mice were cultured with IFN-γ in the absence (*A*) or presence (*B*) of LPS and then fixed. T cells (2×10^4) were added to the fixed macrophage cultures with or without an optimal concentration (0.5–10 μM) of peptide antigen and the proliferative response was measured. (For clones D1.5 and D10, macrophages were incubated with antigen prior to fixation due to the absence of a defined peptide antigen.) Where indicated, recombinant murine IL-1α (rIL-1, 10 pg/ml) or anti-IL-1α antibody (10 ng/ml) was added. Each value represents the mean of triplicate determinations.

B-Cell Antigen Presentation to T_H1 and T_H2 Clones. The capacity of B cells to function as APCs for T_H1 and T_H2 clones was first examined by using live, irradiated splenic B cells. The B cells were routinely fractionated on a density gradient and the high-density cells were selected. This population has been shown to consist of "resting" B cells in contrast to low-density, "activated" B cells (17–19). The B cells were irradiated with either 900 or 3000 rads γ-radiation before coculture with T-cell clones. Low-dose irradiation stops B-cell proliferation without compromising APC function. High-dose irradiation completely inhibits B-cell APC function but not the APC function of macrophage or dendritic cells and is therefore useful as a measure of the purity of the B-cell preparation (17). As shown for a representative experiment (Fig. 3), the high-density B cells induced significant antigen-specific proliferative responses in both T_H1 and T_H2 clones after 900 rads of irradiation but not after 3000 rads.

In further experiments, splenic B cells fixed after various modes of activation *in vitro* were used as APCs. Previous reports (18, 19) had shown that the resting B cell must be activated to function in antigen presentation. A more recent study in our laboratory (C.M.H. and E.R.U., unpublished data) defined conditions by which B cells could be activated with lymphokines to stimulate a primary mixed lymphocyte reaction. B cells served as stimulators of the mixed lymphocyte reaction when first cultured with anti-immunoglobulin (anti-Ig) and IFN-γ. We examined similar B-cell preparations for their capacity to stimulate T_H1 and T_H2 proliferative responses. Results obtained with the T_H1 clone A.E7 are shown in Fig. 4*A*. Resting B cells fixed immediately after isolation were completely ineffective as APCs. In contrast, B cells cultured with anti-Ig and IFN-γ for 48 hr before fixation consistently induced optimal T_H1 proliferation. T_H1 cells cultured with fixed B cells stimulated with anti-Ig alone or with anti-Ig plus IL-4 gave intermediate responses. B cells stimulated with IFN-γ alone, IL-4 alone, or anti-Ig plus TNF-α gave background responses (data not shown). Consistent with results for fixed macrophages (Fig. 2), addition of anti-IL-1α antibody had no effect on T_H1 responses. In experiments where we measured B-cell expression of class II MHC molecules, the levels expressed after treatment with anti-Ig or anti-Ig plus IFN-γ were equivalent and were ≈30% of that induced by anti-Ig plus IL-4 (data not shown). Therefore, the enhanced presenting capacity of B cells activated with anti-Ig plus IFN-γ was not mediated by increased class II MHC molecules or mIL-1.

Fixed B cells activated so as to present antigen to T_H1 clones were ineffectual APCs for T_H2 clones (Fig. 4*B*). Thus, the T_H2 clone CIG12 was unresponsive even at antigen concentrations 100-fold greater than required for an optimal response to fixed LPS-stimulated macrophages (Fig. 2). Addition of exogenous recombinant IL-1 reversed the T_H2 unresponsiveness. Essentially identical results were seen with the entire panel of T_H1 and T_H2 clones.

DISCUSSION

In this study we compared the APC requirements of $CD4^+$ T-cell clones representing T_H1 and T_H2 subsets. Our results indicate striking differences in APC requirements for T_H1 and T_H2 proliferation that are not limited to a given APC lineage

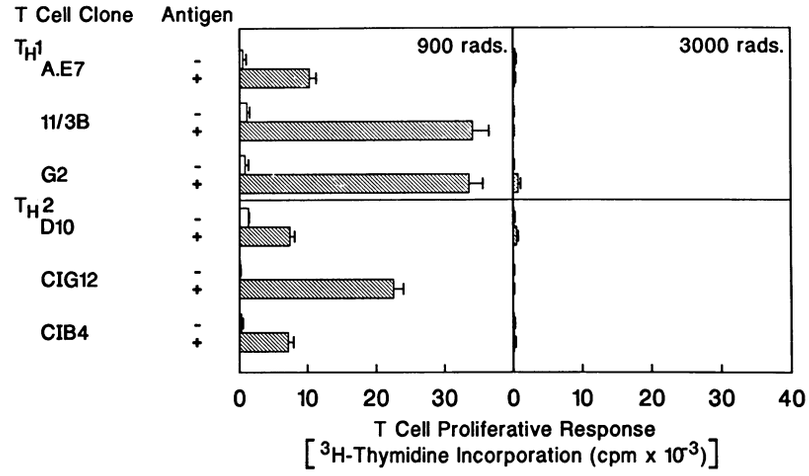

FIG. 3. T_H1 and T_H2 proliferative responses to differentially irradiated resting B cells. High-density splenic B cells (2×10^5) were treated with 900 or 3000 rads prior to culture with T cells (2×10^4) in the absence (−) or presence (+) of relevant antigen. The mean proliferative response of triplicate cultures is shown.

(i.e., macrophages or B cells) but rather to the state of activation of the APCs, particularly regarding the display of distinct costimulator signals. The growth of T_H2 but not T_H1 cells was strictly dependent on the expression of IL-1 by the APCs. This is in agreement with the results of Kurt-Jones *et al.* (6) and Greenbaum *et al.* (20), who examined proliferative responses to lymphokines. Macrophages stimulated to express high levels of mIL-1 were potent APCs for T_H2 clones, whereas those bearing equivalent levels of class II MHC molecules but lacking mIL-1 failed to stimulate. A monoclonal antibody to IL-1α inhibited completely the response that could be reconstituted with exogenous IL-1.

Our results indicate that fixed splenic B cells failed to stimulate T_H2 unless exogenous IL-1 was added; in contrast,

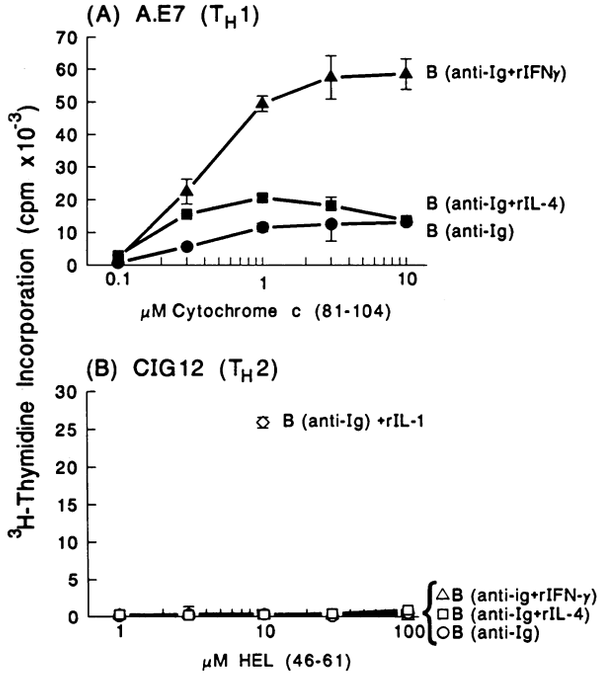

FIG. 4. Differential stimulation of T_H1 and T_H2 clones by fixed B cells. Splenic B cells (2×10^5), fixed after culture for 48 hr with the indicated stimuli, were cocultured with 2×10^4 A.E7 (T_H1) (*A*) or CIG12 (T_H2) (*B*) T cells and a range of peptide antigen concentrations. T-cell proliferative responses were measured and are expressed as the mean of triplicate determinations. r, Recombinant.

live B cells were effective APCs for T_H2 clones. In this regard, we had reported (10) that $CD4^+$ T cells induced mIL-1 on macrophages either by direct contact during antigen presentation or by releasing a lymphokine, now identified as TNF (C.T.W. and E.R.U., unpublished data). Similar phenomena apparently take place in B cells (21). However, the precise nature of the lymphokines from T_H2 cells that may induce mIL-1 is not known. Regardless, preliminary results indicate that the levels of mIL-1 induced in B cells are much lower than in macrophages. Although the experiment of Fig. 3 failed to provide differences in presentation between macrophages and live B cells to T_H1 or T_H2 cells, it is likely that under more stringent conditions the macrophages should be the most efficient APCs for T_H2 cells, based on their higher production of IL-1.

While T_H1 clones did not require IL-1 for growth, they depended on a costimulatory activity not constitutively expressed and best demonstrated in the experiments using fixed B cells as APCs (Fig. 4). B cells stimulated with anti-Ig and IFN-γ had strong stimulatory activity in a primary mixed lymphocyte reaction (C.M.H. and E.R.U., unpublished data). This activity was unrelated to the levels of class II MHC molecules or IL-1. We now find a similar result in the antigen stimulation of T_H1 cells. A non-IL-1 costimulator important for the activation of T cells had been proposed (22, 23). As yet none of these activities has been characterized. We do not know whether the activity induced by anti-Ig and IFN-γ on B cells is the same as that found in the Con A- or *Listeria*-activated macrophages or that reported by others. It will be of interest to determine whether the T_H1 costimulator is, like IL-1, a growth factor or a mediator of cell adhesion as suggested by Krieger *et al.* (24). It will be of further interest to determine its role in the regulation of T-cell tolerance as proposed by Jenkins *et al.* (23).

Whether a predominantly T_H1 or T_H2 response is elicited *in vivo* may depend on many variables, among which the antigen and the APC are probably the most critical. The physiochemical properties of the antigen will determine whether it permeates all tissue microenvironments, whether it has intrinsic properties that readily induce costimulators, and its requirements for uptake and processing by APCs. Concerning the APCs, the levels of class II MHC molecules, processed antigen, and in particular the costimulator expressed will be essential in determining the success of their interaction with one or the other subset of T cells. Clearly a combination of several factors will need to be assessed to

establish which APC is most effective for a T_H1 or a T_H2 response.

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