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The Th1/Th2 Nature of Concurrent Immune Responses to Unrelated Antigens Can Be Independent

Nahed Ismail and Peter A. Bretscher

We tested the independence hypothesis, namely that the Th1/Th2 nature of concurrent immune responses, generated in the same secondary lymphoid organ to non-cross-reacting Ags, can be independently determined. Some infectious agents and some adjuvants contain modulatory molecules that affect the Th1/Th2 nature of immune responses in a non-Ag-specific manner. We therefore excluded infectious agents as Ags and the use of adjuvants to generate immune responses. We first show that the dose of xenogeneic RBC administered i.v. determines the Th1/Th2 nature of the splenic immune response. Low doses generate a virtually exclusive Th1 response, whereas a higher dose induces either a mixed Th1/Th2 or a predominantly Th2 response, and stimulates the production of specific Abs. We immunized individual mice simultaneously with a low dose of one kind of xenogeneic RBC and with a higher dose of another non-cross-reacting xenogeneic RBC and assessed the Th1/Th2 nature of the immune responses generated in the spleen to each kind of RBC. The Th1/Th2 nature of the response to each RBC in doubly immunized mice was indistinguishable from that of the corresponding immune response in singly immunized mice. We discuss the significance of our findings for understanding immune class regulation, and the possible reasons why such independence is not always seen. *The Journal of Immunology, 1999, 163: 4842–4850.*

Observations exemplify the importance of the class of immunity generated upon infection as to whether the infection is contained. In some cases, such as leishmaniasis, tuberculosis, and leprosy, a cell-mediated, Th1 response provides optimal protection (1–3). In others, such as infection by *Tricuris muris* and *Ascaris* (4, 5), an Ab, Th2 response is most beneficial to the host. It seems likely that the immune system incorporates mechanisms that normally favor the generation of an optimally protective immune response. For example, a cell-mediated, Th1 response is probably optimally protective against *Mycobacterium tuberculosis*, the pathogen responsible for tuberculosis, and disease is often associated with an immune response that has an Ab, Th2 component (6). A large majority of individuals infected with *M. tuberculosis* do not suffer from overt disease, presumably due to the generation of a protective, predominantly cell-mediated, Th1 response (2). However, pathogens may have evolved mechanisms to subvert the generation of the most effective response, thereby leading to chronic and progressive infections. Such an ability would provide the pathogen with opportunities to spread through the host population, thus conferring upon such variants a selective advantage.

It would seem that concurrent infections are likely to often occur under natural conditions, even if these are subclinical. In this case, the well being of an individual would require that the Th1/Th2 nature of the immune responses to such concurrent infections be independently determined. For example, it would be disadvantageous if the generation of a Th2 response to *Ascaris lumbricoides* normally involved the production of so much IL-4 that a concurrent response to *M. tuberculosis* was modulated to have a substantial Ab, Th2 component, thus leading to tuberculosis. Reports in the literature demonstrate that responses to one Ag can sometimes affect the Th1/Th2 nature of the response to unrelated Ags. An overwhelming infection to *Schistosoma mansoni*, associated with a Th2 response, can bias the response to sperm whale myoglobin or to influenza virus toward the Th2 pole (7–9), whereas immunization with Bacille Calmette-Guérin, leading to a predominantly cell-mediated response, can bend the response to SRBC toward a cell-mediated mode (10). These examples attest to the interdependency of the Th1/Th2 nature of concurrent responses. It has also been suggested that the increase in allergies among people worldwide may be due to a decrease in infections by mycobacteria, as such infections modulate responses to unrelated Ags toward a Th1 pole (11). In contrast, patients infected with *Microfilaria* generate a predominantly Th2 response, but *Mycobacterium*-sensitized lymphocytes of these patients produce IFN-γ and not IL-4 on stimulation with purified protein derivative of *M. tuberculosis*, as occurs with the *Mycobacterium*-sensitized lymphocytes of uninfected individuals (12). This suggests that the Th1/Th2 nature of concurrent immune responses can be independent. We think that the interdependence of the Th1/Th2 nature of concurrent immune responses to two non-cross-reacting Ags reflects a pathological rather than a physiological situation, involving either an overwhelming immune response to one of the Ags, or the action of immunomodulatory molecules produced by the pathogen. It could also be argued that responses generated in the same lymphoid organ are interdependent, whereas those that are generated by Ag exposure through different routes, such as intestinal exposure and by i.v. administration, might predominantly generate responses in different secondary lymphoid organs that are more independent. We test in this study the most extreme form of the independence hypothesis, namely that the Th1/Th2 nature of the immune response to two non-cross-reacting Ags, generated in the same secondary lymphoid organ, can be independent. Our results support the validity of this
hypothesis, and we discuss the implications of these findings for understanding the mechanism of immune class regulation.

Materials and Methods

Mice
CBA/J mice were obtained from the animal colony at the Department of Microbiology and Immunology, University of Saskatchewan (Canada). Mice, aged 8–12 wk, were employed and were of the same sex within each experiment.

Antigens
Suspension of either SRBC or chicken RBC (CRBC)\(^3\) in Alsevers solution was obtained from animals housed at the University of Saskatchewan. Blood cells were always washed three times and resuspended in Leibovitz L-15 medium (LM) before use.

Immunization
Mice were immunized i.v. with 0.2 ml of LM containing xenogeneic RBC (XRBC), as indicated in the text. Control mice received LM only. Mice were sacrificed and their spleens were harvested at either 3, 4, 5, 6, or 7 days after immunization, and the immune responses were assessed as described below.

\(^3\) Abbreviations used in this paper: CRBC, chicken RBC; DTH, delayed-type hypersensitivity; ELISPOT, enzyme-linked immunospot; FGG, fowl gamma globulin; KLH, keyhole limpet hemocyanin; LM, Leibovitz medium; PFC, plaque-forming cell; pTh, precursor Th; XRBC, xenogeneic RBC.

ELISPOT assay for Ag-specific cells producing IFN-γ or IL-4
Single cell suspensions from the spleen of control and immunized mice were prepared as described previously (13). We employed a variation of the ELISPOT assay (14, 15) to detect Ag-specific IFN-γ- and IL-4-producing cells. The conditions under which we conducted this assay, to be described in detail elsewhere (16), optimize the detection of Ag-specific cytokine-producing cells and ensure that the assay is linear. Briefly, 96-well nitrocellulose-button-culture plates (Polyfiltronics, Rockland, MA) were coated with purified anti-IFN-γ or anti-IL-4 Abs (PharMingen, San Diego, CA) by adding 100 µl/well of Ab at 1.25 µg/ml in 1 M bicarbonate buffer at pH 9.6 and incubating at 4°C overnight. The plates were blocked with 100 µl of RPMI medium containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 U/ml) for at least 1 h before addition of spleen cells. Spleen cells were plated in 100 µl of medium at two densities, 1 × 10⁶ and 5 × 10⁵ cells/well, in the presence of an additional 1 × 10⁶ spleen cells, respectively, from naive, unimmunized mice. Thus, the total number of cells was a constant at 2 × 10⁶ cells/well. The addition of this number of normal spleen cells was found to be necessary to ensure that the number of Ag-dependent spots observed was proportional to the number of immunized spleen cells plated. When required, 10 µl of a 10% suspension of the XRBC was added to the appropriate wells. The number of ELISPOT-forming cells generated in the presence and absence of the Ag by the spleen cells of each mouse was assessed in triplicate. The plates were incubated at 37°C for 8 h and then washed thoroughly with PBS containing 0.05% Tween-20 (PBST). The spots were developed by adding 100 µl of the appropriate biotinylated anti-cytokine Ab (PharMingen), at a concentration of 1.25 µg/ml, and the plates were incubated overnight at 4°C. After thorough washing with PBST, 100 µl of alkaline phosphatase-streptavidin (Jackson ImmunoResearch, West Grove, PA), at a concentration of 0.2

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\text{FIGURE 1. The Th1/Th2 phenotype of the day 5 response following immunization with SRBC or CRBC depends on the Ag dose. Naive CBA mice were immunized i.v. with the indicated numbers of RBC. The number of RBC-specific IFN-γ (filled bars) and IL-4 (open bars)-producing cells per 10^6 spleen cells was assessed using the ELISPOT assay in three independent experiments employing four mice per group.}
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mgl/ml in PBS-Tween, was added to each well. Plates were incubated at room temperature for 1.5 h, and washed with deionized-distilled H2O. The spots were developed by addition of 100 µl of 1/5 dilution in 0.1 M Tris/0.1 M NaCl/0.05 M MgCl2 buffer of nitroblue tetrazolium chloride (8.75 mg/ml), and 5-bromo-4-chloro-3-indolyl phosphate, toluidine (9.4 mg/ml), in 67% (v/v) DMSO, as instructed by the manufacturer (Boehringer Mannheim, Mannheim, Germany). The reaction was stopped after 15 min by washing with deionized-distilled dH2O. The spots were counted once the plate was dry, using a dissecting microscope.

T cell subset depletion

T cells were depleted using the appropriate mAb and complement, as previously described (17).

Measurement of the humoral response

The number of cells producing Ab to CRBC or SRBC was determined by enumerating the number of plaque-forming cells (PFC) using the standard assay for detection of single Ab-producing cells (18). Indirect PFC were obtained by adding rabbit anti-mouse Ig serum to optimally enhance IgG PFC formation.

Results

The Th1/Th2 nature of the immune response to XRBC, administered i.v. without adjuvant, depends upon the Ag dose

We wished to employ a system in which immunization with a nonreplicating Ag of nonmicrobial origin could induce different classes of immune response when administered without adjuvant. Observations in the literature show that XRBC can induce either a humoral or a cell-mediated response depending upon the dose administered (19, 20). We therefore immunized mice i.v. with different numbers of either SRBC or CRBC, and assessed the number of Ag-specific IFN-γ- and IL-4-producing cells in the ELISPOT assay. In the absence of Ag, primed spleen cells produce up to 6 spots/106 plated cells in the IFN-γ assay and up to 10 spots/106 plated cells in the IL-4 assay. Nonsensitized cells produce a similar number of spots independently of the presence of Ag. We only report in this study the Ag-dependent number of spots generated (number of spots in presence of Ag minus number of spots in absence of Ag) per 106 viable white cells plated.

The results of three experiments in which the number of cytokine-producing cells was assessed 5 days following immunization with different doses of SRBC or of CRBC are shown in Fig. 1. It can be seen that the spleen of most mice given a high dose of SRBC (4 × 108) contains a larger number of Ag-specific IL-4-producing cells than the number of Ag-specific IFN-γ-producing cells, i.e., a response with a very substantial Th2 component (Fig. 1A). In contrast, immunization with lower doses of SRBCs (4 × 106 (Fig. 1B) and 4 × 105 (Fig. 1C)) leads to virtually exclusive Th1 responses, with predominance of IFN-γ-producing cells. Similar findings were made with CRBC (see Fig. 1, D, E, and F).

The number of Ag-specific IgM- and IgG-producing cells generated in the spleen was also determined in these experiments. The observations from three experiments employing the two Ags are shown in Fig. 2. The highest dose (4 × 108) induces substantial Ab production (see Fig. 2, A and C), whereas such production is minimally induced by the lower doses (4 × 106; see Fig. 2, B and D).

The Th1/Th2 nature of the immune response at different times following immunization

It may not be valid to conclude from these observations that low doses of XRBC induce a predominantly Th1 response and higher doses a mixed Th1/Th2 response, as the Th1/Th2 nature of the immune response can evolve with time (21, 22). We therefore
examined the kinetics of appearance of Ag-dependent IFN-γ- and IL-4-producing cells in the spleen following immunization with different doses of XRBC (see Fig. 3). The number of Ag-dependent IL-4-producing cells becomes more dominant with time after immunization with a high dose of SRBC (Fig. 3A), whereas the response generated by a low dose of SRBC only has a substantial Th1 component at all time points (Fig. 3B). Our observations on the response to a high dose challenge are consistent with previous reports showing that a cell-mediated response proceeds the production of Ab (21, 22). The observations recorded in Fig. 3 are representative of two independent experiments.

**IFN-γ and IL-4 are produced mainly by CD4+ T cells**

The experiments presented in Fig. 4 were designed to further examine whether RBC-dependent cytokine-producing cells generated in mice immunized with either a low or high dose of SRBC are mediated by T cells of the CD4+ or the CD8+ phenotype. Depletion of CD8+ T cells had only a small effect in mice immunized with either a low dose of SRBC only has a substantial Th1 component at all time points (Fig. 3B). Our observations on the response to a high dose challenge are consistent with previous reports showing that a cell-mediated response proceeds the production of Ab (21, 22). The observations recorded in Fig. 3 are representative of two independent experiments.

**The Th1/Th2 nature of concurrent immune responses can be independently determined**

We wished to determine whether the Th1/Th2 nature of concurrent immune responses to non-cross-reacting Ags could be independent. We therefore first ascertained whether CRBC and SRBC cross-reacted as assessed in the ELISPOT assay. Spleen cells from CRBC-immunized mice produced a substantial number of spots in the ELISPOT assay in the presence of CRBC, but not SRBC, and vice versa (data not shown). We concluded that these two Ags were not significantly cross-reactive and are therefore appropriate Ags for testing the independence hypothesis.

To test this hypothesis, we examined the Th1/Th2 nature of the immune responses in three groups of mice, 5 days after immunization. One group received only a low dose of XRBC, the second group received only a high dose of a non-cross-reacting XRBC, whereas the third group received both kinds of XRBCs at the same dose as the mice receiving only one kind of RBC. We found that the Th1/Th2 nature of the immune responses to the two XRBCs in double-immunized mice was indistinguishable from that of the responses in singly immunized mice. Immunization with a low dose
of CRBC or SRBC produced a predominantly Th1 response in both doubly and singly immunized mice (see Fig. 5, A and C), whereas immunization with a high dose of SRBC or CRBC produced a mixed or predominantly Th2 response in both singly and doubly immunized mice (see Fig. 5, B and D). These observations are from one of three independent experiments showing similar results employing five mice per group.

We also examined the number of Ab-producing cells generated in singly and doubly immunized mice. Mice doubly immunized with a high dose of one RBC and with a low dose of another RBC

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**FIGURE 5.** The Th1/Th2 nature of the immune responses to non-cross-reacting XRBC is independently determined. Individual CBA mice were immunized either with both a low dose of one RBC and a high dose of another, non-cross-reacting RBC, or with only one kind of RBC at the indicated dose. The number of Ag-specific IFN-γ and IL-4-producing cells generated in the spleens of doubly and singly immunized mice was assessed 5 days after immunization using the ELISPOT assay.

**FIGURE 6.** The generation of IgM- and IgG-producing cells is independent of concurrent immune responses. Mice were either immunized with both a low dose of one RBC and a high dose of another, non-cross-reacting RBC, or immunized with only one kind of RBC, as indicated. RBC-specific IgM- and IgG-producing cells were measured 5 days after immunization.
produced a similar and substantial number of IgM and IgG Ab-producing cells to the high dose challenge as seen in singly immunized mice (see Fig. 6, A and C). Similarly, the response to a low dose challenge of one RBC was unaffected by a concurrent challenge with a high dose of another RBC (see Fig. 6, B and D).

The Th1/Th2 nature of an immune response is not affected by the presence of an ongoing immune response occurring at the time of immunization

It could be argued that the observed independence of the Th1/Th2 nature of concurrent immune responses is not surprising, as the cytokine environment at the time of initiation of the immune response is critical in affecting the Th1/Th2 nature of the immune response. In doubly immunized mice, no CRBC-specific T cells have been activated at the time of immunization with a high dose of CRBC that might affect, by the cytokines they produce, the response to a low dose of SRBC. Maximal generation of Ag-specific cytokines occurs at about day 5 postimmunization (see Fig. 3). We therefore further tested the independence hypothesis by examining the effect of an ongoing immune response to one RBC on the response to a challenge of another RBC. Mice were immunized with either a low or a high dose of one XRBC, and 5 days later, they and unprimed mice were challenged with a high or low dose of the other XRBC. We compared the Th1/Th2 nature of the immune responses in these two groups of mice, 5 days after the second RBC challenge. We also ascertained that the first immunization generated a response of the expected nature by sacrificing some of the mice at the time of the second immunization.

The results presented in Fig. 7 show that the Th1/Th2 nature of the immune response to a high dose of either CRBC or SRBC is independent of whether the mice had received a previous immunization with a low dose of the other non-cross-reacting RBC (Fig. 7, A and B). The immune response to a low dose of either SRBC or CRBC is also independent of whether the mice had received a previous challenge of a high dose of the other RBC (Fig. 7, C and D). These observations are representative of three separate experiments. An examination of Ab responses showed that the immune response to a high or low dose of one kind of XRBC was minimally affected by prior low or high dose priming respectively with another non-cross-reacting RBC (see Fig. 8).

Discussion

A strong Th2 response is generated in mice with an overwhelming infection of Schistosoma mansoni. This can cause the down-regulation of Th1 responses to other Ags, resulting, for example, in delayed clearance of vaccinia virus (8). In contrast, we show in this study that the Th1/Th2 nature of concurrent, nonoverwhelming immune responses to non-cross-reacting Ags can be independently determined. This implies that an individual under normal circumstances can contain simultaneous infections that require immune responses of different Th1/Th2 phenotype. We discuss in this work how such independence might be achieved and how it might be subverted.

There are several contemporary models for what events determine whether a common precursor Th (pTh) cell is activated by Ag to produce Th1 or Th2 progeny. These include the strength of the TCR signal (23, 24), the nature and/or strength of costimulatory signals (25, 26), the nature of the cytokines present in the environment (27, 28), and the responsiveness of particular subsets of T cells to particular cytokines (29). Recent observations suggest a role for the cell cycle in controlling Th cell differentiation (30, 31). All of these factors are likely to contribute to a full description of the decision criterion that determines whether Th1 or Th2 cells are predominantly generated. However, we feel that other factors, less frequently discussed in the current literature, are central to understanding this decision criterion and the independency of the generation of immune responses as reported in this study.
The activation of resting pTh cells requires, at least under some circumstances, an interaction of this T cell with other Ag-specific T cells, and this interaction is mediated by the operational recognition of linked epitopes (32, 33). For example, T cells primed to fowl γ globulin (FGG) can help the generation of delayed type hypersensitivity (DTH)-mediating Th1-like cells specific for a XRBC if the FGG is coupled to this XRBC, but not if the FGG is present but coupled to an irrelevant, non-cross-reacting RBC (32). We believe this requirement for the recognition of linked epitopes is essential to understanding how the independence of the Th1/Th2 nature of concurrent immune responses is achieved, and so a consideration of the mechanisms by which this might occur is pertinent.

The evidence for and against the mechanism we favor has been discussed (34, 35). It postulates that pTh activation requires a first step involving presentation by professional APC such as dendritic cells, and a second step in which Ag-specific, resting B cells take up the Ag, process it, and subsequently present the peptides of the Ag on their class II MHC molecules. Inducible costimulatory molecules are expressed on the B cells when effector Th cells recognize these MHC-bound peptides, and such induced B cells can now activate pTh cells specific for another MHC-bound peptide derived from the same Ag. This mechanism accounts for the recognition of linked epitopes in the T cell-T cell collaboration observed to be required for the activation of pTh cells (32, 33).

This operational requirement for recognition of linked epitopes is likely to be relevant to understanding the old generalization that different Ags have different abilities to induce cell-mediated responses in the form of DTH and Ab. An Ag with only a few foreign sites or, in modern terms, able to give rise to only a few foreign peptides recognized by CD4+ T cells, can only induce a cell-mediated response, whereas an Ag with many foreign sites can induce either a predominantly cell-mediated or Ab response, depending upon the exact circumstances of immunization (36, 37). There is considerable evidence for this generalization (38). For example, the very foreign CRBC and SRBC employed in this study can induce Th1 or mixed Th1/Th2 responses depending upon the dose administered, but the less foreign rat RBC (rats and mice both being rodents) induce a virtually exclusive Th1 response even when administered at high doses (N. Ismail and P. A. Bretscher, unpublished observations). This generalization, if valid, must mean that the immune system has a means of evaluating the number of foreign sites on an Ag, or, in modern terms, of foreign peptides recognized by CD4+ T cells. What kind of decision criterion could account for the importance of the number of such peptides, derivable from the Ag, in determining the Th1/Th2 nature of the response that the Ag induces? It has been suggested that a quantitative formulation of the model briefly outlined above for pTh activation can accomplish this (35, 37). This quantitative formulation states that the degree of expression of inducible costimulatory molecules (39) on the Ag-specific B cell determines whether a pTh cell is activated to give rise to Th1 or Th2 cells; in the presence of few pTh/Th cells and poor expression of the inducible costimulatory molecules, Th1 cells are generated, whereas in the presence of more pTh/Th cells and better expression, Th2 cells are generated. This hypothesis is called the threshold hypothesis (35, 40).

This hypothesis is consistent with our previous observations showing that more unprimed T cells are required to generate Ab than cell-mediated responses under experimental conditions in which the nature and the dose of the Ag are kept constant (41, 42). Our more recent results show that it is the number of unprimed CD4+ T cells that determines the Th1/Th2 nature of the response under similar experimental conditions, i.e., where the nature and dose of the Ag are held constant (N. Ismail and P. A. Bretscher, unpublished observations). Furthermore, it is known that the generation of a potent Ab response to an Ag renders the animal unresponsive for the induction of DTH to this Ag. This refractoriness has been called humoral immune deviation. Humorally immune mice bear Ag-specific CD4+ Th cells that can inhibit the induction...
of DTH, and this inhibition also involves the recognition of linked epitopes, i.e., of peptides recognized by CD4+ T cells and derived from the same immunogenic entity. Thus, CD4+ T cells from an animal humorally immune to a protein Ag P can inhibit the generation of CD4+ DTH-mediating T cells specific for a XRPC in the presence of the conjugate P-XRPC, but not when both Ags are present, but in unlinked form (43).

The Th1/Th2 nature of the response to the diverse peptides derived from a single protein, or to the peptides derived from the many proteins of an intracellular parasite, tends to be similar. Thus, for example, infection with few Leishmania major parasites results in a Th1 response to all the components of a parasite (44). This similarity in the Th1/Th2 nature of the response is said to reflect coherence in the regulation of the immune response (35). Coherence is explicable in terms of the threshold hypothesis, as the response to the different CD4+ T cell-recognized peptides of the Ag is interdependent.

In conclusion, we relate our findings of independence to the requirement for the recognition of linked epitopes in the activation of pTh cells in the following manner. Immunization with a high dose of SRBC results in Ab production, the generation of a mixed Th1/Th2 response, and SRBC-specific T cells that can suppress cell-mediated immunity. These T cells act by the recognition of linked epitopes, and so will not affect the Th1 response generated following immunization with a low dose of a non-cross-reacting Ag. Somewhat similarly, the requirement for the recognition of linked epitopes in the activation of pTh cells, as envisaged in the threshold hypothesis, allows us to understand how the Th1/Th2 nature of the immune response to non-cross-reactive Ags can be independent.

This view then leaves us with the question of how might such independence be subverted? We can envisage at least two general mechanisms. The first is that pathogens produce molecules that have immunomodulatory functions independent of their Ag specificity. Thus, it is well recognized that at least some mycobacteria can modulate the immune responses to nonmycobacterial Ags toward a cell-mediated pole (10, 11), and it is plausible that the stimulation of IL-12 production by APC by mycobacterial products may be involved (45). Such activity may well contribute to the adjuvant function of microbial products. We consider that the characterization of conditions under which Ag recognition by nonlinked mechanisms can regulate immune responses may provide clues as to how independence of responses might be subverted. For example, it is known that in the presence of very high concentrations of the Ag keyhole limpet hemocyanin (KLH), anti-KLH Th cells can help the in vitro anti-hapten B cell response in the presence of a conjugate, h-c, in which C and KLH do not cross-react (46). A suggested mechanism for how such nonlinked help might occur seems plausible. At very high Ag concentrations, the anti-hapten B cells take up sufficient amounts of KLH by non-receptor-mediated pinocytosis, so that the exposed B cells express KLH-derived, class II MHC-bound peptides on their surface. Such B cells can be helped to produce anti-hapten Ab by anti-KLH Th cells. It would seem plausible, given the central role of B cells as envisaged in the threshold hypothesis, that similar events may explain the lack of independence of responses when the modulating response is an overwhelming parasitic infection.

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