

Do T Helpers 1 and 2 Recognize Different Class II MHC Molecules? Humoral and Cellular Immune Responses to Soluble Allergen from *Aspergillus fumigatus* Asp f2

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Cellular signals leading to T helper (Th)1/Th2 shift are not well known. Here we demonstrate that Th1 possibly recognizes peptides presented by the IE molecule of MHC class II while Th2 is activated by the recognition of peptides presented by the IA molecule. BALB/c mice immunized with Asp f2 developed stable IA-restricted Th2 immune response to the 12th day after immunization, as analyzed by IL-2 production. On the contrary, early Th0 cells did not secrete IL-2 upon Asp f2 stimulation but did produce a high level of IL-2 if stimulated in the presence of anti-IA Abs. This effect of anti-IA Abs on early Th0 cells was both MHC IE and CD4⁺ cell restricted. In vivo blocking of Asp f2 peptide presentation by the IA molecule led to the formation of antigen-specific cytotoxicity as demonstrated using immune splenocytes as effector cells and Asp f2 loaded P815 cells as targets. © 2001 Academic Press

Key Words: *Aspergillus fumigatus*; allergens; Th1/Th2 switch; MHC class II molecules.

INTRODUCTION

The immune response to a specific antigen must induce an appropriate set of effector functions that can eliminate the particular pathogen involved in the infection. The central event in the generation of cell-mediated or humoral immune responses is the activation and differentiation of naive CD4⁺ T helper cells (Th) into Th1 or Th2 subsets which exert most of their helper function through different sets of secreted cytokines (1). The Th1 subset is responsible for the activation of cell-mediated immunity and cytotoxic CD8⁺ T lymphocytes (CTLs), while the Th2 subset primarily functions as a helper for B cell activation (2). The Th1 subset secretes IL-2 and INF- γ , through which it activates CTLs and macrophages and also switches B cells to synthesize IgG2a. The Th2 subset secretes IL-4, IL-5, IL-6, and IL-10 and is responsible for the formation of both IgG1 and IgE antibodies to extracellular patho-

gens such as freeliving bacteria and helminthic parasites. Th2 cells also mediate allergic reactions (3, 4).

The direction in which the naive CD4⁺ cells will differentiate is dependent on their first encounter with antigens. The factors that determine this differentiation are not fully understood although the cytokine environment that is present during differentiation of antigen-primed Th cells is thought to determine the subset which is emerging (5). We demonstrate here that Th1 and Th2 subsets can be generated under the same environment but are likely to recognize peptides presented by different class II MHC molecules.

It is well known that CD4⁺ T cells recognize peptides in the context of MHC class II molecules (6). Peptide–MHC class II complexes are formed in the APC which internalize the antigen, process it in the endocytic pathway, and transport the complexes to the cell surface. Peptides from the extracellular pathogens will be presented mainly by MHC class II molecules although there are accumulating data demonstrating the accessibility of phagocytized antigen through the conventional cytosolic processing pathway leading to MHC class I presentation (7–9). The proteins from intracellular pathogens such as viruses are processed in the cytosolic pathway and presented on the cell membrane mainly with MHC class I molecules. As these pathogens are also partially phagocytized by APC during extracellular virus cell-to-cell spreading, this leads to the formation of complexes between MHC class II molecules and antigenic peptides which activate the antigen-specific Th1 subset (10). Results presented here demonstrate the possible existence of Th1 and Th2 precursors which recognize different MHC class II molecules.

The model antigen Asp f2 which we have used in this study originates from the fungus *Aspergillus fumigatus* and is implicated in the severe forms of allergic response to *A. fumigatus* proteins, namely allergic bronchopulmonary aspergillosis. Asp f2 and other *A. fumigatus* proteins are soluble antigens and when injected by intrana-

sal, intraperitoneal, or subcutaneous routes induce a Th2 type of immune response (3, 4, 11, 12).

MATERIAL AND METHODS

Animals

Ten- to 12-week-old inbred BALB/c (H-2^d) mice bred at the Institute of Bioorganic Chemistry Animal Department, Moscow, were used in this study. The protocol for experimental animals used was approved by the ethics committee of the Institute of Bioorganic Chemistry, RAS, Moscow, Russia.

Antigen and Peptides

Recombinant *Asp f2*, a 37-kDa major antigen from the fungus *A. fumigatus*, was cloned in *Escherichia coli* and purified by Ni chromatography (13).

Antigen Treatment

In the first experiment the dynamics of MHC class II restriction of the immune response to *Asp f2* was studied. Mice (three to five per group) were immunized subcutaneously with 100 μ g of *Asp f2* in incomplete Freund's adjuvant in the right hind footpad. Mice were sacrificed on days 6, 12, and 25 after immunization and the blood and draining popliteal lymph nodes (LN) were obtained.

In the second experiment the role of class II MHC molecules in the induction of the immune response *in vivo* was studied. Two groups of mice were immunized with 50 μ g of *Asp f2* as described above. Group I received additionally 10 injections of normal murine IgG while group II received 10 injections of anti-IA^d Ab (MK-D6, ATCC, HB-3). The injections were given subcutaneously near the lymph node draining the site of immunization during 5 days (two per day) after antigen immunization. Each injection contained 10 μ g of IgG. The immunization schedule was repeated again after a month's interval. Mice were sacrificed 6 days after the last immunization.

Spleen and Lymph Node Cell Cultures

Single cells from the spleen and LN were obtained by pressing cells through a stainless steel wire mesh. The LN cells were washed and cultured at 2×10^5 cells in 0.2 ml of RPMI 10 medium (Gibco-BRL) with or without MK-D6 Ab. Cells cultured in a 96-well flat-bottom tissue culture plate (Linbro, UK) were incubated at 37°C in a 5% CO₂ incubator. As LN are poor in APC, additional untreated peritoneal exudate cells (2×10^3 /well) obtained from the same immune animals were added. Various concentrations of *Asp f2* antigen were

added and culture supernatant containing Ab against MHC molecules was added to a final concentration of 25% of the culture medium. Cultures of LN cells were incubated for 24 h to analyze for IL-2 production or for 72 h to estimate cell proliferation. The IL-2-dependent CTLL-2 cell line was used to determine IL-2 in 24-h LN cell culture supernatants. Proliferation of LN was studied by [³H]TdR incorporation during the last 12 h of the culture. Anti-IA^d, IE^d (Pharmingen), and purified anti-H-2K^d Ab (ATCC, HB 159) were used in control cultures. Purification of CD4⁺, CD8⁺ subpopulations was carried out by cytotoxic complement-dependent lysis using supernatants of hybridomas GK1.5 (ATCC, TIB 207) and NS1 producing anti-L3T4 and anti-Lyt 2 Ab, respectively, and fresh rabbit serum was added as a source of complement. The results of IL-2 production are shown as an index of stimulation calculated as $IS = X:Y$, where X is the cpm in cultures with antigen and Y is the cpm in cultures without antigen but with antibodies where is indicated. Standard deviation of SI (dSI) is calculated as $dSI = (YdX - XdY)/Y^2$, where X and Y are means explained above and dX , dY are their standard deviations.

Cytotoxic Test

Cytotoxic activity was analyzed using splenic T cells as effectors and P815 murine mastocytoma cells (H-2^d) as targets. Different concentrations of splenic T cells depleted from adherent and B cells by a panning procedure (rabbit anti-murine IgG serum, Institute of Bioorganic Chemistry, Moscow) and treated with mitomycin C (40 μ g/ml) (Sigma, St. Louis, MO) to prevent proliferation were mixed with 2×10^4 P815 cells/well, centrifuged for 5 min at 500 rpm to form a pellet, and incubated overnight. To analyze the number of proliferating P815 cells 1 μ Ci/well [³H]TdR was added to the cultures during the last 12 h. The residual level of T cell proliferation was estimated in separate wells and subtracted from the cpm in wells containing both T cells and P815. Index of inhibition (II) was calculated as

$$II = 1 - [\text{cpm}_{\text{exp}}/(\text{cpm}_{\text{P815}} + \text{cpm}_{\text{spleen}})],$$

where cpm_{exp} is the mean cpm in triplicate cultures with mixed effectors and targets, $\text{cpm}_{\text{spleen}}$ is the cpm in cultures containing effector cells only, and cpm_{P815} is the cpm in cultures containing target cells only. Standard deviation for II was calculated as explained above. To analyze antigen-specific cytotoxicity, 10^6 P815 cells were incubated in 1 ml with 20 μ g *Asp f2* for 3 h at 37°C in 5% CO₂ before being used as targets. Natural killer activity was analyzed using P815 not loaded with the antigen.

Cytokine mRNA Transcription in Splenocytes

The total RNA was extracted from 10^7 LN cells stimulated for 3 days with $10 \mu\text{g/ml}$ of *Asp f2*. LN cells from group II were stimulated in the presence of anti-IA Ab. Cultured LN cells were washed and the RNA was isolated by the guanidine isothiocyanate phenol chloroform single-step method (14). cDNA was prepared from mRNA with Maloney's murine leukemic virus reverse transcriptase (Bion, Moscow, Russia). The specific genes were amplified by PCR using sense and anti-sense primers of IL-4 and IFN- γ as described before (15). β -Actin primers were similarly used as a housekeeping control. The PCR products were studied on a 2% agarose gel and the amplified bands were visualized after staining with ethidium bromide. The size of the amplified fragments was determined by comparison with a 1-Kb standard DNA ladder and the β -actin band.

RESULTS

Mature Immune Response to Soluble Allergen *Asp f2* Is IA Restricted

In the first series of experiments the dynamics of the restriction of immune response to *Asp f2* was studied. For this, LN cells were obtained on days 6, 12, and 25 after immunization and stimulated with *Asp f2* in the presence of additional APC. To estimate the class II MHC restriction of the early and mature primary immune response, Ab against the IA molecule were added to some cultures. Figure 1 demonstrates that on day 6 anti-IA Ab stimulated IL-2 production from *Asp f2*-specific T cells, but the subsequent mature immune response was found to be IA restricted as the addition of anti-IA Ab abrogated IL-2 production completely in a wide range of antigen concentrations. Different dose-dependent curves were observed when the dynamics of immune response was studied. We could demonstrate three peaks on day 14 and a single peak on day 25 (Figs. 1A and 1B), and this may be due to T cell epitope selection at an earlier stage of the mature immune response. This effect, as well as the stimulatory effect of anti-IA^d antibodies on IL-2 production and T cell proliferation, was reproduced for LN cells and splenic T cells. Similar results were also observed when splenic adherent cells or purified B cells were used as APC (data not shown).

To analyze in more detail the effect of anti-IA Ab stimulation during the early immune response the effect of Ab to both class II MHC molecules and class I MHC molecule K^d was studied. LN cells were obtained on day 4 after immunization to study the earliest immune response. No proliferation was observed in cultures stimulated with the antigen only, but prolifera-

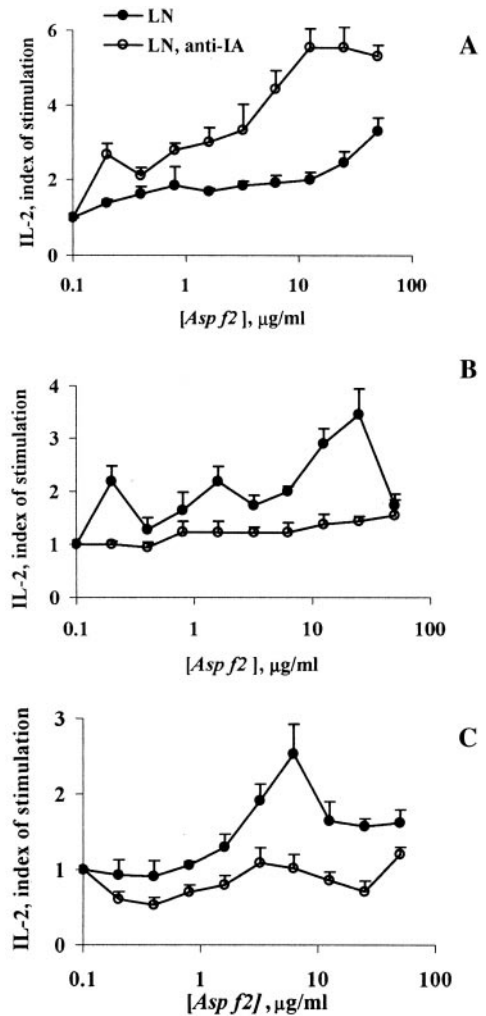


FIG. 1. Formation of IA-restricted immune response to soluble antigen *Asp f2*. Mice (BALB/c) were immunized with $100 \mu\text{g}$ of *Asp f2*. LN cells were collected on days 6 (A), 12 (B), and 25 (C) and stimulated *in vitro* for IL-2 production with *Asp f2* only (solid circles) or in the presence of anti-IA Ab MK-D6 (open circles). Adherent spleen cells were added to the cultures as a source of additional APC. In this figure and in all other figures the data from a single representative experiment are shown. The same stimulation effect is observed when splenic T cells are studied or if B cells are used as APC. Error bars represent standard deviation of the mean calculated as described under Material and Methods.

tion increased significantly in cultures stimulated with the antigen in the presence of anti-IA Ab and not in the presence of anti-IA/IE or anti-K Ab (Fig. 2). No IL-2 production was found in these cultures, possibly due to it being below detectable levels or to high levels of consumption. The removal of CD8⁺ cells had no effect on this stimulatory effect and the removal of CD4⁺ cells abrogated this effect. We have concluded that some CD4⁺ T cells are able to recognize antigenic peptides presented by the IE molecule during the early immune response to soluble antigen, a fact demon-

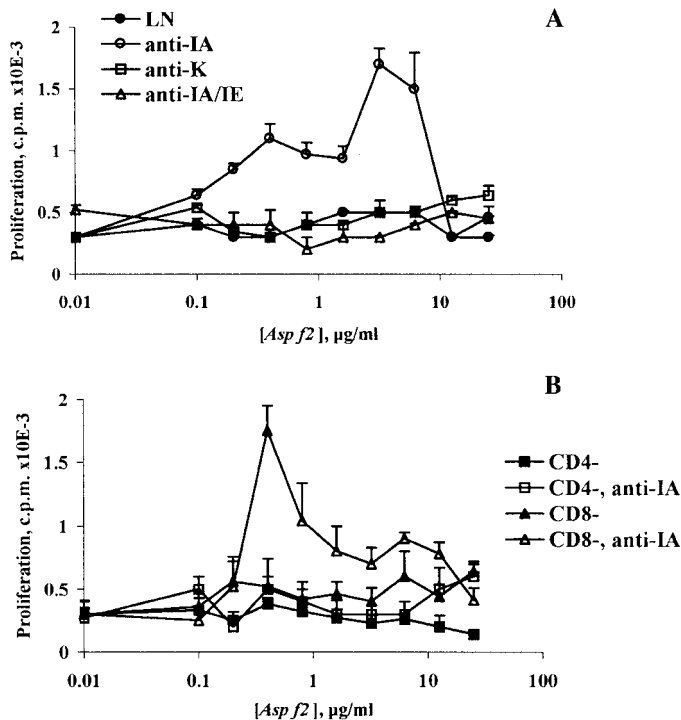


FIG. 2. The role of CD4⁺ and CD8⁺ T cells in the early immune response to *Asp f2*. Total LN cells (A) or purified CD4⁺ and CD8⁺ LN cells (B) from mice immunized 4 days in advance were stimulated *in vitro* with *Asp f2* only (solid symbols) or in the presence of Ab to MHC molecules (open symbols). Proliferation was estimated at 72 h by [³H]TdR incorporation during the last 12 h of the culture.

strated by the addition of anti-IA Ab. Their activation is possible only if the IA molecule on APC is blocked with Ab. This stimulation was never observed in the mature primary immune response (Figs. 1B and 1C). Earlier it was shown that the immune response to *A. fumigatus* allergens is mediated by the Th2 subset (3, 4, 11, 12). So, we have hypothesized that early CD4⁺ cells able to recognize peptides presented by the IE class II MHC molecule could belong to the Th1 subset or their precursors. To study this possibility we have blocked the IA-restricted immune response *in vivo* by injecting anti-IA^d Ab during 5 days after immunization of mice with *Asp f2*.

Induction of Th1 Subset to Soluble Antigen *Asp f2*

To block the IA-restricted immune response, mice immunized with *Asp f2* received additionally 10 injections of anti-IA Ab (group II) or control normal murine IgG (group I) locally around the site of immunization. This immunization protocol was repeated twice to accumulate more antigen-specific cells. Th1 and Th2 functions of LN and spleen cells were studied after the second immunization. The immune response of LN cells from mice of group I was IA restricted (Fig. 3) in

the same manner as that shown in Fig. 1. Thus, the administration of normal murine IgG has no influence on MHC class II restriction. On the contrary, the effect of anti-IA Ab was clearly observed as the immune response in group II mice was IE restricted. The addition of murine IgG in control cultures did not affect IL-2 production (data not shown).

Natural killer cells (NK) and antigen-specific cytotoxic CD8⁺ cell activity were studied by cytotoxic assay using MHC-matched target cells (P815). Both NK and antigen-specific activity were observed in spleen T cells of group II mice (Fig. 4). Some antigen-specific cytotoxicity was also observed in group I, indicating that the humoral immune response is accompanied by some cellular immunity with a probable regulatory function.

It is known that Th1 and Th2 produce overlapping but different sets of lymphokines during the immune response. IFN- γ and IL-4 are the most often used markers of Th1 and Th2, respectively. Using RT-PCR we have studied the transcription of these marker cytokines, IFN- γ and IL-4, in both groups. The produc-

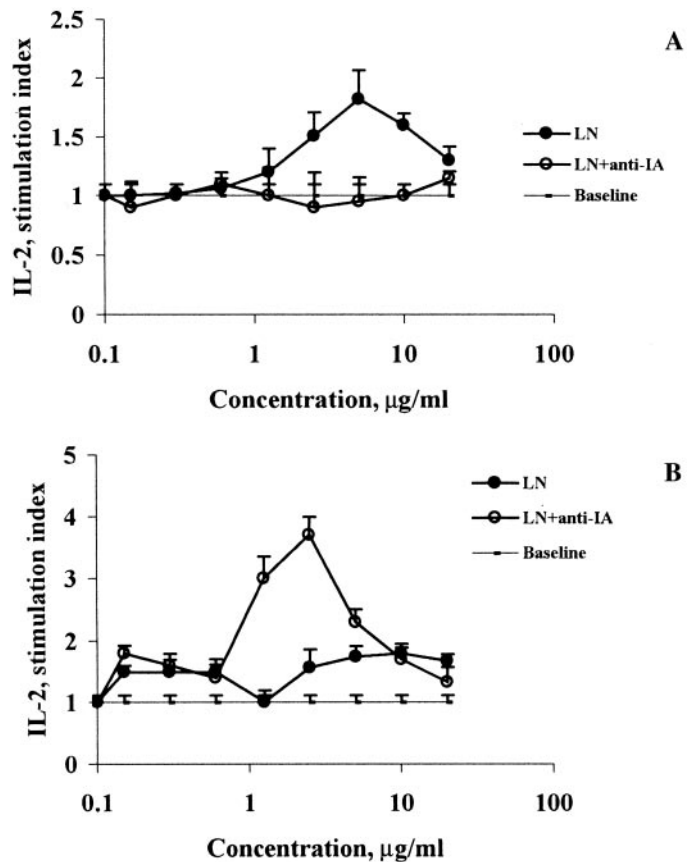


FIG. 3. IE-restricted immune response in mice treated with anti-IA Ab. Two groups of mice were immunized with *Asp f2* and treated with normal murine IgG (group I) or anti-IA Ab (group II). LN cells from group I (A) or II (B) were collected on day 6 after the second immunization and restimulated *in vitro* with *Asp f2* for IL-2 production without (solid circles) or in the presence of anti-IA Ab (open circles).

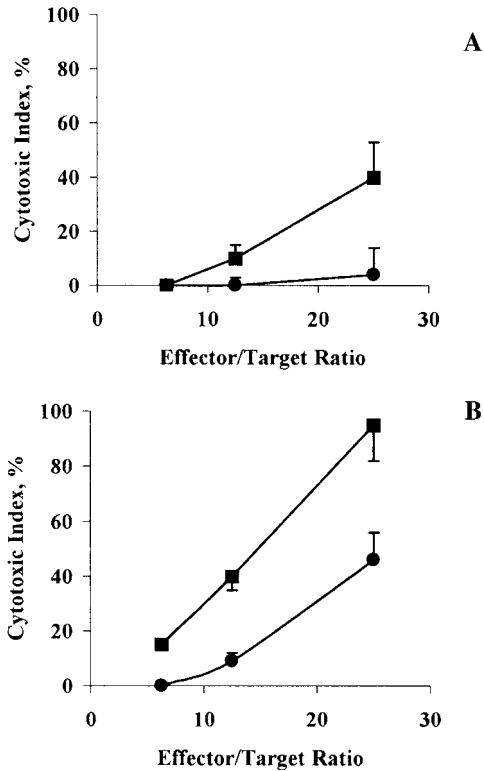


FIG. 4. Natural killer activity and antigen-specific cytotoxicity in mice treated with anti-IA Ab. Two groups of mice were immunized with *Asp f2* and treated *in vivo* with normal murine IgG (group I) or anti-IA Ab (group II). Natural killer activity (A) or antigen-specific cytotoxicity (B) in group I (solid circles) or group II (solid squares) was estimated using spleen T cells purified by panning as effector cells and untreated P815 mastocytoma cells (A) or loaded with *Asp f2* for 3 h (B) as target cells for NK cells or CTLs, respectively.

tion of only IL-4 was observed in group I while IFN- γ was the predominant cytokine with a small amount of IL-4 observed in group II (Fig. 5). Although RT-PCR is a semiquantitative method, we have used two cycles of repeated PCR to detect very low levels of IFN- γ mRNA in group I. No detectable amount of IFN- γ was demonstrated (data not shown).

DISCUSSION

CD4⁺ T cells undergo maturation in the thymus, where they are selected by interaction with the MHC class II molecule. It is not clear whether Th cells in mice can recognize both class II molecules or only one. It is also not known whether IA and IE molecules differ in their TCR contact residues. The exact roles of different MHC class I and II molecules of mice and humans are not precisely known. The majority of T cell epitopes from soluble proteins such as OVA, lysozyme, and influenza hemagglutinin are IA restricted; however, IE-restricted T cell epitopes are also found in soluble proteins (16). Here we have shown that the immune response to soluble allergen

Asp f2 is completely IA restricted until the 10th–14th day after immunization (Fig. 1). However, during the early immature immune response, on days 4–9 after immunization a stimulatory effect of anti-IA Ab was observed. We hypothesize that this effect can be attributed to the process of Th type selection leading to Th2 immunity. This assumption was based on the facts that: (i) some CD4⁺ cells were able to produce IL-2 when the IA molecule was blocked (Figs. 1 and 2); (ii) the stimulation detected was IE restricted, as Ab to both IA and IE molecules blocked the stimulation completely (Fig. 2); and (iii) IE-restricted CD4⁺ cells were not activated unless anti-IA Ab were added, suggesting that they were down-regulated by IA-restricted CD4⁺ cells (Fig. 1). As only two subsets of CD4⁺ cells are known to mediate most immune reactions in mice, we hypothesize that Th cells recognizing peptides presented by the IE molecule could belong to the Th1 subpopulation. The possibility that these cells are activated only during the early immune response indicates that the process of Th1/Th2 selection is completed by the 10th–14th day after immunization with the soluble antigen.

To study the role of IA and IE molecules in the formation of the immune response to soluble protein *in vivo*, we have treated mice with anti-IA Ab during the 5 days after immunization, creating a condition where the IA molecule is blocked. Th1/Th2 functions in both groups were studied. IA-restricted IL-2 production by immune LN cells dominated in the control group (Fig. 3), while IE-restricted immune response was found in the experimental group, which was significantly stimulated by the presence of anti-IA Ab *in vitro*.

To study the function of Th1 we have analyzed NK cell activity and antigen-specific cytotoxicity. Both functions demonstrate higher Th1 activity in the experimental group (Fig. 4). Th1/Th2 activation was also supported by the specific lymphokine production. In the control group only IL-4 production was noted, while in the experimental group a low amount of IFN- γ was detected.

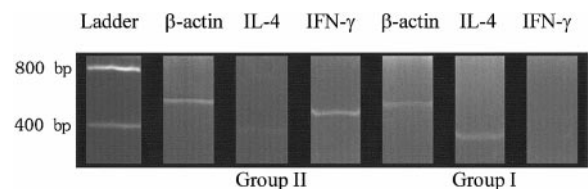


FIG. 5. Lymphokine production in mice treated with anti-IA Ab. Two groups of mice were immunized with *Asp f2* and treated *in vivo* with normal murine IgG (group I) or anti-IA Ab (group II). Ten million LN cells were cultured *in vitro* with 10 μ g/ml of *Asp f2* and 5 U/ml of hrIL-2 with no anti-IA Ab (group I) or with anti-IA Ab (group II) for 3 days. The mRNA was extracted and analyzed using RT-PCR. LN cells from group I produced only IL-4 mRNA, while in group II mainly IFN- γ and some amount of IL-4 mRNA was found. The DNA ladder bands correspond to 800 and 400 bp; β -actin, 539 bp; IL-4, 400 bp; IFN- γ , 462 bp.

The antigen-specific cytotoxic activity found in both groups supports the earlier demonstrated phenomenon of cross priming, leading to class I MHC presentation of peptides originating from soluble antigens (7–9). In addition, we have demonstrated that the maturation of antigen-specific CTL depends on CD4⁺ cells recognizing the class II MHC molecule, in particular the IE molecule.

The most intriguing question involves the IA-restricted suppression of the Th1 subset during the immune response initiation (Fig. 1). This may be linked with the cooperation among Th1, Th2, and CD8⁺ cells recognizing different MHC molecules. There are no data demonstrating the principal difference between the two MHC class II molecules. Hence, it is very likely that in the endosomal compartment both MHC class II molecules are loaded with antigenic peptides with the same efficacy. As a consequence, not much difference could be predicted in the number of IA and IE complexes on the surface of APC when the antigen is processed either by the endosomal or by the cytosolic pathway. However, clear differences discriminating antigen processing by these two pathways could be observed in the total amount of peptide–MHC class I molecule complexes. When soluble protein undergoes endosomal processing, high amounts of peptide–class II complexes versus low amounts of peptide–MHC class I molecule complexes will be observed. Contrary to this, when viral proteins are processed by the cytosolic pathway high amounts of MHC class I complexes loaded with viral peptides are found. Hence, it could be assumed that a high density of peptide–IA molecule complexes expressed on the APC surface is enough to activate the Th2 subset with a consistent down-regulation signal to the Th1 cells. On the contrary, the signal mediated in Th1 cells by the interaction with peptide–IE molecule complexes is not sufficient to activate Th1 cells, and in order to be activated Th1 cells need some additional signal, probably from CD8⁺ cells recognizing peptide–MHC class I complexes on the surface of the same APC.

The conclusion derived from the results presented in this report is rather indirect. If proven to be correct, it will assume that Th1 and Th2 originate not from the common precursor pTh0 but from different naive precursors pTh1 and pTh2, which are selected in the thymus by recognition of different MHC class II molecules. Another consequence of this hypothesis is that the activation of pTh1 is dependent on the interaction of CD8⁺ cells with the same APC.

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