Preferential Recognition of Hepatitis B Nucleocapsid Antigens by Th₁ or Th₂ Cells Is Epitope and Major Histocompatibility Complex Dependent†

DAVID R. MILICH,^{1*} DARRELL L. PETERSON,² FLORIAN SCHÖDEL,³ JOYCE E. JONES,¹
AND JANICE L. HUGHES¹

Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037¹; Department of Biochemistry, Virginia Commonwealth University, Richmond, Virginia 23298²; and Institut National de la Santé et de la Recherche Médicale, Unité 80, Hôpital Edouard Herriot, F-69437 Lyon Cedex, France³

Received 3 November 1994/Accepted 2 February 1995

Regulatory T-helper (Th) cells have been categorized into two functional subsets, Th₁ and Th₂ cells, which produce distinct lymphokines. In general, Th₁ cells mediate cellular immune responses and Th₂ cells mediate humoral immunity. Recent serological studies suggest that the Th₁-Th₂ balance may be relevant in acute and chronic hepatitis B virus (HBV) infections. The purpose of this study was to determine the potential of the nucleocapsid antigens (Ag) (hepatitis B core and e Ags [HBc/eAg]) of HBV to preferentially elicit either a Th₁ or a Th₂ dominant response. For this purpose, H-2 congenic B10.S and B10 mice were immunized with HBc/eAg, and Ag-specific T-cell proliferative responses, T-cell helper function, and T-cell cytokine production were analyzed. The results indicated that B10.S mice preferentially develop a Th₁-like response whereas B10 mice preferentially develop a Th₂-like response after immunization with HBc/eAg. Furthermore, the preferential Th₁ and Th₂ response patterns were reproduced when 12-residue peptides representing the dominant HBc/eAg-specific T-cell sites for B10.S (peptide 120-131) and B10 (peptide 129-140) mice were used as immunogens. Therefore, the combination of the T-cell site recognized and the major histocompatibility complex restricting element can in large part determine the Th phenotype of the HBc/eAg-specific T-cell response. Other factors that influenced Th phenotype were the presence of exogenous cytokines, Ag structure, and tissue distribution.

Early studies of in vivo immune responses demonstrated independent regulation of cellular and humoral immunity. The recent elucidation of distinct subsets of regulatory CD4⁺ T-helper (Th) cells designated Th₁ and Th₂ cells (39) has significantly advanced our understanding of the earlier studies. The Th subsets are largely defined by a unique pattern of cytokine secretion such that Th₁ cells tend to produce interleukin-2 (IL-2), gamma interferon (IFN-γ), and tumor necrosis factor beta, whereas Th₂ cells produce IL-4, IL-5, IL-6, IL-10, and IL-13 (43). The differences in cytokine secretion explain the functional dichotomy between Th₁ cells, which enhance cellular immunity, and Th₂ cells, which are best adapted to helping B cells produce antibody. Furthermore, products of Th₁ and Th₂ cells can negatively regulate the reciprocal subset because IFN-γ, produced by Th₁ cells, inhibits Th₂ proliferation, and IL-10 and IL-4, produced by Th₂ cells, inhibit synthesis of Th₁ cytokines and block development of Th₁ cells (9, 12, 13, 19, 55). Lymphokines also influence the isotype of antibody responses. Human and murine immunoglobulin E (IgE) and murine IgG1 responses are promoted by IL-4, whereas IFN-γ enhances IgG2a responses in the mouse (11).

The pathophysiologic significance of distinct Th₁-Th₂ phenotypes has primarily emerged from studies of protozoan parasitic infections (see reference 57 for a review). For example, the outcome of *Leishmania major* infection is dependent upon which Th subset is preferentially activated: a Th₁-mediated

response leads to resistance, and a Th₂-mediated response results in susceptibility (25). Importantly, the pattern of resistance and susceptibility can be altered by various immunologic manipulations. For example, treatment of nonhealer BALB/c mice with anti-IL-4 converts a Th₂ pattern to a Th₁ pattern of cytokine expression which confers resistance (6), whereas depletion of IFN-γ in resistant C3H/HeN mice leads to susceptibility (1).

In addition to the *Leishmania* system, the importance of differential Th₁-Th₂ activation has been observed in a number of bacterial (7, 16, 51), fungal (48), and helminth (44, 61) infections. These studies are consistent with the concept that Th₁-mediated responses (i.e., IFN-γ) are protective against infections by organisms that are resident within cells and Th₂-mediated responses (i.e., antibody) are protective against infections by extracellular microbes (i.e., nematodes) (43). The role of preferential Th₁-Th₂ induction in the control of viral infections has not been as extensively examined. Recent serologic studies comparing patients with acute, self-limited hepatitis B virus (HBV) infection and symptomatic patients chronically infected with HBV revealed significantly greater antibody responses in the chronically infected patients (27) even though cellular immune responses appear to be significantly greater in acutely infected patients (10). Furthermore, approximately 50% of asymptomatic chronic HBV-infected patients demonstrated antibody production in the absence of immune-mediated liver disease (26). We have proposed that the predominance of different Th subsets in acute and chronic HBV infection may explain these observations (26, 27). For example, the predominance of nucleocapsid-specific Th cells of the Th₂ subset during chronic infection would favor antibody production over cell-mediated immunity, whereas predomi-

* Corresponding author. Mailing address: Department of Molecular Biology, CAL-2, The Scripps Research Institute, 10666 N. Torrey Pines Rd., La Jolla, CA 92037. Phone: (619) 554-3643. Fax: (619) 554-8494.

† Publication 8771-MB from the Scripps Research Institute.

nance of the Th₁ subset in acute infection would favor cell-mediated immunity, which may result in efficient viral clearance mechanisms.

This current report represents an initial study to examine (i) whether the nucleocapsid antigens (Ags) of HBV (i.e., hepatitis B core and e Ags [HBcAg and HBeAg]) can elicit preferential recognition by Th₁ or Th₂ cells and (ii) the variables that may influence such recognition in a murine model. For this purpose, *H-2* congenic B10.S (*H-2^s*) and B10 (*H-2^b*) mice were immunized with various forms of the HBV nucleocapsid antigens, and antigen-specific T-cell proliferative responses, T-cell helper activity, and T-cell cytokine production were monitored. Indeed, evidence of preferential Th₁-Th₂ recognition of the HBV nucleocapsid antigens was observed, and the influences of T-cell epitope, major histocompatibility complex [MHC] genotype, tissue distribution, antigen structure, and the addition of exogenous cytokine on Th₁-Th₂ induction were examined.

MATERIALS AND METHODS

Mice. C57BL/10 (B10) (*H-2^b*) and *H-2* congenic B10.S (*H-2^s*) and (B10 × B10.S)_F₁ mice were obtained from the breeding colony of The Scripps Research Institute. The transgenic (Tg) lineage designated B10.S-Tg31e, which expresses HBeAg (9 to 13 ng/ml) in the serum, was produced as described previously (31). The F₁-Tg31e mice (e/+) were produced by breeding B10.S-Tg31e mice homozygous for the HBeAg transgene with non-Tg B10 mice.

Recombinant proteins and synthetic peptides. The HBV core gene encodes two polypeptides. Initiation of translation at the first start codon (AUG) results in a 25-kDa precore protein that is secreted as HBeAg after removal of 19 residues of the leader sequence and 34 COOH-terminal amino acids. Initiation of translation at the second AUG leads to the synthesis of a 183-amino-acid, 21-kDa protein that assembles to form 27-nm particles that comprise the virion nucleocapsid (HBcAg). Although HBeAg and HBcAg are serologically distinct, these Ags are cross-reactive at the level of Th-cell recognition because of the fact that they are colinear throughout most of their primary sequence. Recombinant HBcAg of the *ayw* subtype was produced in *Escherichia coli* and purified as described previously (52). A truncated version of HBcAg (residues 1 to 149) was produced in *E. coli*. This material is particulate but expresses both HBcAg and HBeAg antigenicity at the level of antibody recognition and is designated HBc/eAg. A recombinant HBeAg corresponding in sequence to serum-derived HBeAg encompassing the 10 precore amino acids remaining after cleavage of the precursor and residues 1 to 149 of HBcAg was produced as described previously (53) and is designated PC-HBeAg. The presence of the 10 precore amino acids prevents particle assembly and PC-HBeAg is recognized efficiently by an HBeAg-specific monoclonal antibody (MAb) but displays little HBc antigenicity (53). An aliquot of truncated HBc/eAg was reduced and denatured by boiling in sodium dodecyl sulfate (SDS)-2-mercaptoethanol (1.0%) and alkylated, the SDS was removed by dialysis, and the material was resolubilized in 3 M urea. This preparation consisted predominantly of monomers (16 kDa) with some dimer formation upon nonreducing polyacrylamide gel electrophoresis and was designated P16. P16 does not bind HBcAg or HBeAg-specific MAbs.

Peptides were synthesized by the simultaneous multiple peptide synthesis method (17) (kindly provided by Richard Houghton, Torrey Pines Institute for Molecular Studies, La Jolla, Calif.). The following HBcAg-derived synthetic peptides representing Th-cell recognition sites were used and designated by amino acid position from the N terminus of HBcAg: 120-131 (IA^a), VSFGV WIRTPPA; 129-140 (IA^b) PPAYRPPNAPIL; 129-140[133A], an alanine substitution at the epitopic residue 133 (37); and 120-140, a 21-mer comprising both T-cell sites. Synthetic IL-4 corresponding to amino acids 24 to 140 of murine IL-4 was generously provided by Ian Clark-Lewis (Biomedical Research Center, University of British Columbia, Victoria, British Columbia, Canada).

Serology. HBeAg was measured in diluted Tg mouse sera by a commercial enzyme-linked immunosorbent assay (ELISA) (HBe EIA; Abbott, Chicago, Ill.), and PC-HBeAg was used as a standard. Anti-HBc and anti-HBe IgG antibody was measured in murine sera by an indirect solid-phase ELISA using HBcAg or PC-HBeAg as the solid-phase ligand as described previously (38). The data are expressed as antibody titer representing the reciprocal of the highest dilution (1/log₂) of sera required to yield a reading three times that of preimmunization sera (38).

In vivo Th-cell assay. Th-cell activity for antibody production was determined by the ability of peptide T-cell sites to prime mice to produce anti-HBc or anti-HBe antibody in vivo after challenge with a suboptimal dose of HBcAg or PC-HBeAg, respectively. Groups of five mice were primed intraperitoneally (i.p.) with 100 µg of peptide in complete Freund's adjuvant (CFA). Control mice were primed with CFA alone. Four weeks after priming, the mice were challenged with suboptimal concentrations of HBcAg (0.08 µg) or of PC-HBeAg (0.5 µg) in

incomplete Freund's adjuvant, and sera were collected for measurement of IgG anti-HBc or anti-HBe 7 and 14 days after challenge.

Adoptive transfer of autoantibody production in HBeAg Tg mice. Non-Tg donor mice were immunized in the hind footpads with 100 µg of the peptide T-cell site 120-131 or 129-140 emulsified in CFA or CFA alone, and draining popliteal lymph node (PLN) cells were harvested after 10 days and cultured with peptide 120-140 (0.2 µg/ml) or purified protein derivative for an additional 3 days. In vitro-activated PLN cells (5 × 10⁶) were then transferred into sublethally irradiated (500 R) HBeAg-expressing Tg recipient mice (33). Sera were collected before and at various times after adoptive transfer and analyzed for HBeAg (autoantigen) and anti-HBe (autoantibody) by ELISA.

In selected experiments, T cells were enriched from the PLN preparation before adoptive transfer, which produced similar results. For convenience and because donor mice were primed with and PLN cells were cultured with peptides that represent T-cell recognition sites on HBcAg- but not HBeAg-specific B-cell sites (36), unselected PLN cells were routinely used to transfer HBcAg-specific T-cell help in adoptive transfer experiments.

T-cell proliferation assay. Groups of at least five mice each were primed with 5 to 20 µg of HBc/eAg or 100 µg of peptide antigens in CFA by hind footpad injection. Ten days after immunization, draining PLN cells were harvested and pooled, and 5 × 10⁵ cells in 0.1 ml of Click's medium were cultured with 0.1 ml of medium containing various concentrations of HBc/eAg, synthetic peptides, or medium alone. Purified protein derivative, which is present in CFA, served as the positive control Ag. Cells were cultured for 96 h at 37°C in a humidified 5% CO₂ atmosphere, and during the final 16 h, 1 µCi of [³H]TdR (6.7 Ci/mmol; Amersham, Arlington Heights, Ill.) was added. The cells were then harvested onto filter strips for determination of [³H]TdR incorporation. The data are expressed as counts per minute corrected for background proliferation in the absence of Ag. The T-cell nature of the proliferation was confirmed by analyzing nylon-wool column-enriched T cells in selected experiments. All T-cell activation experiments were performed at least three times, and representative experiments are depicted.

Determination of Ag-specific cytokine production. Groups of five mice each were immunized with various Ag preparations emulsified in complete or incomplete Freund's adjuvant either in the hind footpads or i.p., and 10 days later PLN cells or spleen cells were harvested, pooled, and cultured (8 × 10⁶/ml) with various concentrations of a series of Ags as described for the T-cell proliferation assay. Culture supernatants (SN) were harvested at 24 h for IL-2 determination and at 48 h for IL-4 and IFN-γ determinations. IL-2 was measured by the ability of SN to stimulate proliferation of the IL-2- and IL-4-sensitive NK-A cell line in the presence of MAb 11B11 specific for IL-4 (40). IL-4 was measured by the ability of SN to stimulate proliferation of the IL-4-sensitive CT.4S cell line (generously provided by William Paul, National Institutes of Health, Bethesda, Md.) (20). IFN-γ was measured by two-site ELISA using MAb HB170 and a polyclonal goat anti-mouse IFN-γ (Genzyme Corp., Boston, Mass.). In an attempt to normalize cytokine measurements, in some experiments data were expressed as reciprocals of the minimum concentrations of Ag necessary to elicit measurable (three times background) amounts of the various cytokines. All cytokine experiments were performed at least three times, and representative data are shown.

RESULTS

T-cell proliferative responses to HBc/eAg and constituent peptides in B10, B10.S, and (B10 × B10.S)_F₁ mice. The comparative T-cell proliferative responses elicited by immunization with the particulate HBc/eAg was determined in B10, B10.S, and (B10 × B10.S)_F₁ mice. Previous studies have identified the dominant HBc/eAg-specific T-cell recognition site in B10 mice as residues 129 to 140 and in B10.S mice as residues 120 to 131 (29). As shown in Fig. 1, immunization with HBc/eAg elicits relatively weak T-cell proliferative responses in B10 mice regardless whether the recall Ag in vitro is HBc/eAg, peptide 120-140, or peptide 129-140, and the 120-131 sequence is not recognized by B10 T cells. In contrast, immunization with HBc/eAg induces vigorous T-cell proliferative responses in B10.S mice, which are focused on the 120-131 sequence. Immunization of (B10 × B10.S)_F₁ mice with HBc/eAg elicits efficient 120-131-specific (IA^s-restricted) T-cell proliferation and relatively weak 129-140-specific (IA^b-restricted) T-cell proliferation (Fig. 1). These data suggest that the B10.S strain is a high responder and that the B10 strain is a relatively low responder to HBc/eAg at the T-cell level. However, if antibody (i.e., anti-HBc) is measured, the B10 strain produces comparable (34) and, in the experiment shown, higher levels of IgG

FIG. 1. Comparative HBeAg-specific T-cell proliferative responses in B10, B10.S, and (B10 × B10.S)_{F1} mice. Groups of five B10, B10.S, and (B10 × B10.S)_{F1} mice were immunized with 10 μg of HBeAg in CFA by hind footpad injection. Ten days later, draining PLN cells were harvested, pooled, cultured (5×10^6 /ml) with the indicated concentrations of HBeAg, peptide 120-140, 120-131, or 129-140, or medium alone, and analyzed for in vitro T-cell proliferation. T-cell proliferation is expressed as [³H]TdR uptake corrected for background proliferation in the absence of Ag (Δcpm). Background proliferation ranged from 550 to 3,750 cpm. Sera were collected before and 10 days after immunization for the determination of anti-HBe antibody titer by ELISA.

anti-HBc antibody than the B10.S strain (Fig. 1; compare data in brackets).

It was also of interest to determine if the disparity in HBc/eAg-specific T-cell proliferation observed between B10 and B10.S mice also existed if peptide 120-140, containing both relevant T-cell sites, was used as the immunogen (Fig. 2). As predicted by the previous results, immunization with peptide 120-140 elicited minimal T-cell proliferation specific for HBc/eAg and the 129-140 sequence within 120-140 in B10 mice. In B10.S mice, the 120-140 peptide was very immunogenic and induced strong T-cell proliferative responses focused on residues 120 to 131 within peptide 120-140 (Fig. 2). Therefore, the peptide immunogen 120-140 reproduced the results obtained with native HBc/eAg, confirming that the enhanced T-cell proliferative response demonstrated by the B10.S strain compared with the B10 strain was at the level of epitope recognition. The differential T-cell proliferative responses of B10 and B10.S mice may reflect differences in the overall avidity of the trimolecular T-cell receptor (TCR)-peptide-MHC interaction. The immunogenicity of peptide antigens has been reported to depend primarily on the affinity of peptide-MHC binding (56). The interaction between the 129-140 T-cell determinant and IA^b may be of lower affinity than the 120-131-IA^s interaction (28, 32). In addition to the greater immunogenicity of 120-131 than of 129-140 in terms of T-cell proliferative responses, the relatively weak immunogenicity of 129-140 is shared by a 129-140[133A] analog which possesses a distinct T-cell fine specificity (i.e., epitope) but the same MHC binding residues (aggreptope) as 129-140 (37) (data not shown). This finding suggests that the reduced immunogenicity of 129-140 is more likely due to inefficient MHC binding as opposed to inadequate TCR interaction. However, a direct comparison between 129-140 and 120-131 in terms of MHC binding is problematic because different MHC class II molecules are involved.

HBc/eAg-specific in vivo Th-cell function in B10 and B10.S mice. One explanation for comparable levels of antibody production to HBc/eAg in B10 and B10.S mice in the context of low and high T-cell proliferation status, respectively, would be a dichotomy between T-cell helper function and T-cell proliferation. Therefore, the dominant HBc/eAg-specific T-cell recognition sites for B10 (residues 129 to 140) and B10.S (residues 120 to 131) mice were examined for the ability to prime T cells capable of providing functional help for in vivo antibody production to HBcAg and HBeAg in (B10 × B10.S)F₁ mice. For

this purpose, an in vivo Th assay was used (35). Mice were primed with the synthetic T-cell determinants in CFA or with CFA alone and challenged in vivo 4 weeks later with suboptimal doses of HBcAg or nonparticulate PC-HBeAg, and serum antibody levels were measured 7 and 14 days after the challenge. This approach requires that the memory Th cells primed by immunization with peptide are recalled by challenge with native Ag, indicating the relevance of the synthetic T-cell site to the native molecule. Using this protocol, the 129-140 peptide was shown to be superior to the 120-131 peptide in priming for both anti-HBe and anti-HBc antibody production in F₁ mice (Fig. 3). In fact, the IA^b-restricted peptide 129-140 was 64-fold more efficient than the IA^s-restricted peptide 120-131 in eliciting in vivo anti-HBe production 7 days after the challenge and 256-fold more efficient 14 days after the challenge with PC-HBeAg. Similarly, peptide 129-140 was 16-fold more efficient than peptide 120-131 in priming anti-HBc antibody responses (Fig. 3). The higher titer and faster kinetics of the anti-HBc responses relative to anti-HBe antibody production and the reduced disparity between B10 and B10.S anti-HBc responses may reflect a lesser requirement for T-cell helper function for antibody production to HBcAg, which can function as a T-cell-independent Ag (34). These data indicate that B10 HBc/eAg-specific T cells proliferate rather poorly in vitro yet can elicit efficient T-cell help for in vivo antibody production, and reciprocally, B10.S HBc/eAg-specific T cells proliferate efficiently but appear less well adapted to providing T-cell help for antibody production. This scenario suggests the possibility that immunization with HBc/eAg or constituent synthetic T-cell sites preferentially elicits Th₂ cells in B10 mice and Th₁ cells in B10.S mice.

A second and possibly more revealing method to discern the T-cell helper potential of 120-131- and 129-140-primed T cells was to examine the ability of peptide-primed T cells to elicit autoantibody production upon adoptive transfer into HBeAg-expressing F₁ Tg mice. (B10 × B10.S)F₁ (+/+) donor mice were primed with peptide 120-131 or 129-140 in CFA or with CFA alone and cultured for 3 days in vitro with 120-140, and 5 × 10⁶ activated T cells were transferred into sublethally irradiated F₁ HBeAg-Tg (e/+) recipient mice. As shown in Table 1, unprimed (CFA only) T cells derived from non-Tg (+/+) donors did not elicit autoantibody (i.e., anti-HBe) upon transfer into HBeAg-Tg recipients. Apparently endogenous serum HBeAg (~10 ng/ml) is not sufficiently immunogenic to

FIG. 2. Comparative 120-140-specific T-cell proliferative responses in B10 and B10.S mice. Groups of five B10 and B10.S mice were immunized with 100 μ g of HBc/eAg-derived peptide 120-140 in CFA by hind footpad injection. Ten days later, draining PLN cells were harvested, pooled, cultured (5×10^6 /ml) with the indicated concentrations of HBc/eAg, peptide 120-140, 129-140, or 120-131, or medium alone, and analyzed for in vitro T-cell proliferation as described for Fig. 1.

FIG. 3. HBeAg-specific in vivo Th-cell function in B10 and B10.S mice. Groups of 5 (B10 $\times$ B10.S) F_1 mice were primed i.p. with 100 μ g of 120-131 or 129-140 in CFA or with CFA alone (0). Four weeks later, primed mice were challenged with suboptimal concentrations of HBeAg (0.08 μ g) or PC-HBeAg (0.5 μ g) in incomplete Freund's adjuvant, and sera were collected for measurement of IgG anti-HBe and IgG anti-HBc prechallenge and 7 and 14 days after the challenge. Antibody titers, expressed as the reciprocal of the highest dilution of sera to yield three times the optical density at 492 nm reading of prechallenge sera, were determined by ELISA.

activate naive HBeAg-specific Th cells. The control of transferring 129-140-primed and in vitro-activated T cells into non-Tg recipients was also performed, and no anti-HBe antibody was produced (not shown). However, the transfer of 129-140-primed and in vitro-activated T cells into $e/+$ Tg recipients elicits significant autoantibody production (i.e., 1:40,960) as early as 2 weeks posttransfer, which is sufficient to neutralize detection of the circulating serum HBeAg (autoantigen) (Table 1). The titer of autoantibody increased to 1:163,840 by weeks 4 and 6. Note that anti-HBe antibody production occurs without immunization with exogenous HBeAg; therefore, endogenous HBeAg serves as the only source of Ag for HBeAg-specific B cells, which are not tolerant in this model. Also note that the autoantibody consists almost exclusively of the IgG1 isotype (1:163,840), with very little IgG2a (1:2,560) or IgG2b (1:2,560) and no IgG3 isotype. This finding suggests that autoantibody production in this Tg model is primarily mediated by HBeAg-specific Th₂ cells because IL-4 enhances IgG1 production whereas IFN- γ enhances IgG2a production (11). In this regard, it is interesting that transfer of peptide 120-131-primed T cells into $e/+$ Tg recipient mice elicits no autoantibody production 2 weeks after transfer and very little at 4 weeks (1:160) and 6 weeks (1:640) after transfer, and no neutralization of circulating HBeAg is observed (Table 1). These data suggest that 120-131-primed T cells are deficient in Th₂ activity compared with 129-140-primed T cells. Interestingly, addition of exogenous IL-4 during the 3-day in vitro culture of 120-131-primed T cells partially reverses the deficiency, and transfer of IL-4-treated 120-131-specific T cells elicits moderate levels of autoantibody production in $e/+$ Tg recipients, which neutralizes circulating HBeAg by approximately 50% 4 to 6 weeks posttransfer (Table 1). This observation suggests that either 120-131-specific precursor Th cells capable of differentiating into Th₂ cells are still present in the draining lymph node 10 days after in vivo priming, that small numbers of 120-131-specific Th cells are of the Th₂ phenotype but require exogenous IL-4 for their expansion, or, less likely, that mature 120-131-specific Th₁ cells can be converted into Th₂ cells. The adoptive transfer results indicate that immunization

with epitope 129-140 (IA^b restricted) efficiently elicits Th₂-like cells capable of mediating in vivo IgG1 autoantibody production, whereas epitope 120-131 (IA^s restricted) elicits T cells that are capable of HBeAg-specific proliferation but do not mediate efficient in vivo autoantibody production (i.e., Th₁-like).

TABLE 1. T-cell transfer of autoantibody production in (B10 $\times$ B10.S) F_1 HBeAg-Tg mice^a

Ag	No. of cells	Time (wk)	Anti-HBe/auto-antibody (1/titer)	Serum HBeAg concn (ng/ml)
CFA	5×10^6	2	0	10.4
		4	0	10.1
		6	0	10.0
129-140	5×10^6	2	40,960	1.2
		4	163,840 (G1) 2,560 (G2a) 2,560 (G2b) 0 (G3)	0
		6	163,840	0
120-131	5×10^6	2	0	10.5
		4	160	12.1
		6	640	12.4
120-131 + IL-4	5×10^6	2	640	11.1
		4	10,240	6.2
		6	10,240	5.7

^a Non-Tg donor mice (+/+) were primed in the hind footpads with CFA alone or with 100 μ g of the synthetic T-cell site 129-140 or 120-131 emulsified in CFA. Draining PLN cells were harvested and cultured in vitro with either purified protein derivative (CFA control) or peptide 120-140 for 3 days. In vitro-activated T cells (5×10^6) were transferred into four to five sublethally irradiated (500 R) HBeAg-Tg recipient mice ($e/+$). Sera were collected before transfer and 2, 4, and 6 weeks after adoptive transfer and analyzed for IgG anti-HBe (autoantibody) and serum HBeAg concentration (autoantigen) by ELISA. The anti-HBe IgG isotype profile was determined by using IgG1, IgG2a, IgG2b, and IgG3-specific second antibodies in the ELISA. IL-4 (20 μ g/ml) was added during the 3-day in vitro culture.

TABLE 2. HBc/eAg-specific cytokine production by B10.S and B10 splenic T cells^a

Strain	Ag	1/minimum Ag concn		
		IL-2	IL-4	IFN- γ
B10.S	HBcAg	3,333 (8.6)	25 (2.9)	100 (4.0)
	PC-HBeAg	125	5.0	100
	120-140	3,333	5.0	62.5
	120-131	3,333	25	25
	129-140	0	0	0
B10	HBcAg	25 (2.1)	3,333 (16)	25 (0.5)
	PC-HBeAg	25	125	5.0
	120-140	5.0	125	0.2
	120-131	0	0	0
	129-140	5.0	125	.04

^a Groups of 5 B10.S or B10 mice were immunized i.p. with 20 μ g of HBc/eAg in CFA, and 10 days later spleen cells were harvested, pooled, and cultured (8×10^6 /ml) in vitro with various concentrations of the Ags indicated. Culture SN were collected for IL-2, IL-4, and IFN- γ determination. The data are expressed as the reciprocal of the minimum Ag concentration required to elicit measurable (three times background in the absence of Ag) amounts of the various cytokines. For example, a reading of 3,333 represents a minimum Ag concentration of 0.0003 μ g/ml required to elicit measurable cytokine production. For comparative purposes, absolute levels of cytokine induced by HBcAg (1.0 μ g/ml) are shown in parentheses in units per milliliter for IL-2 and IL-4 and in nanograms per milliliter for IFN- γ . This analysis was performed three separate times, and the data shown are representative.

Direct measurement of HBc/eAg-specific T-cell cytokine production in B10.S and B10 mice. Because the preceding experiments suggested the possibility that the inverse relationship between HBc/eAg-specific T-cell proliferation and antibody production relates to the differential induction of Th phenotypes, splenic T-cell cytokine production in B10.S and B10 mice immunized with HBc/eAg was determined. Spleen cells of HBc/eAg-immunized B10.S and B10 mice were cultured with the HBc/eAg-derived Ags listed in Table 2, and SN were harvested at 24 h for IL-2 determination and at 48 h for IL-4 and IFN- γ measurements. As shown in Table 2, HBc/eAg-primed B10.S splenic T cells cultured with HBcAg, PC-HBeAg, 120-140, and 120-131 produce IL-2 efficiently at low in vitro Ag concentrations (i.e., 1/minimum Ag concentration of 3,333 equals an in vitro HBcAg concentration of 0.0003 μ g/ml). Only peptide 129-140 failed to elicit IL-2 production. Similarly, B10.S splenic T cells primed with HBc/eAg secreted IFN- γ quite efficiently at relatively low in vitro concentrations of all Ags except 129-140, the IA^b-restricted peptide. Although HBc/eAg-primed B10.S splenic T cells produced IL-4 upon culture with the panel of Ags, the minimum in vitro concentrations of the Ags required were from 666.6 to 25 times higher than the minimum concentrations required to elicit IL-2 production and from 20 to 4 times higher than those required to elicit IFN- γ with the exception of peptide 120-131, which elicited IL-4 and IFN- γ equally. Therefore, HBc/eAg-primed splenic T cells from B10.S mice predominantly, but not exclusively, produce the Th₁-type cytokines IL-2 and IFN- γ upon in vitro stimulation with this panel of Ags. In contrast, the cytokine profile of HBc/eAg-primed splenic T cells from B10 mice is characteristic of a Th₂-type response in that IL-4 is the predominant cytokine and is produced at least 25 times more efficiently than either IL-2 or IFN- γ with respect to the minimum in vitro concentrations of the Ags required to elicit cytokine production. Only peptide 120-131 failed to stimulate HBc/e-primed B10 splenic T cells (Table 2). Note that IL-2 and IFN- γ are produced at lower levels than IL-4 but are not

absent in HBc/eAg-primed B10 splenic T cells. These data indicate that the polyclonal T-cell responses to HBc/eAg immunization in B10.S and B10 mice are composed of a mixture of HBc/eAg-specific Th₁ and Th₂ cells; however, there is a clear predominance of IL-2- and IFN- γ -secreting Th₁ cells in B10.S spleen and reciprocally a predominance of IL-4-secreting Th₂ cells in the spleens of B10 mice. Splenic HBc/eAg-specific cytokine production in these two H-2 congenic strains is consistent with and may explain the inverse relationship between T-cell proliferation and antibody production observed in the B10 and B10.S strains.

Tissue distribution of HBc/eAg-specific Th₁ and Th₂ cells. During the course of these studies, it was very difficult to detect Ag-specific IL-4-producing T cells in draining PLN cells even in HBc/eAg-immunized B10 mice. Therefore, we compared the draining lymph node and splenic T-cell compartments in terms of HBc/eAg-specific cytokine production in B10 mice (Fig. 4). B10 mice were immunized either in the hind footpads or i.p. with HBc/eAg, and PLN cells or spleen cells, respectively, were harvested and cultured with HBc/eAg and peptide 129-140 in vitro for subsequent cytokine analysis of SN. Because no differences in IL-2 production were noted between the lymph node and splenic compartments, only levels of IL-4 and IFN- γ production over a range of Ag concentrations (0.0003 to 25 μ g/ml) are shown. As shown in Fig. 4A, B10, HBc/eAg-primed PLN cells produce little (HBc/eAg-induced) or no (129-140-induced) IL-4 in culture. This was the most vigorous response observed, and on many occasions we were unable to detect any HBc/eAg-specific IL-4 production by B10 PLN T cells. In contrast, HBc/eAg-primed PLN T cells produced IFN- γ in an Ag dose-dependent manner upon culture with HBc/eAg or 129-140 in vitro. The B10 splenic compartment demonstrated another pattern (Fig. 4B). Splenic B10 HBc/eAg-primed T cells produced IL-4 efficiently upon in vitro activation with HBc/eAg or 129-140 throughout the dose-response curves. Splenic T cells from B10 HBc/eAg-immunized mice quantitatively produced IFN- γ when cultured with HBc/eAg but failed to do so when cultured with peptide 129-140 (Fig. 4B). Therefore, there are clear differences between the tissue distribution of HBc/eAg-specific Th₁ and Th₂ cells, which may reflect preferential priming locations or preferential migration patterns.

Effect of immunogen structure on induction of a Th₁ or Th₂ dominant response. An inherent advantage in studying T-cell recognition of the HBV nucleocapsid Ags is the availability of multiple structural forms of the same T-cell sites. For example, HBcAg is a 27-nm particle, PC-HBeAg is nonparticulate but probably multimeric, P16 represents a monomeric polypeptide, and peptides 120-131 and 129-140 represent the minimum sequence necessary for T-cell recognition. It was therefore of interest to determine if the structure of the immunogen influenced Th phenotype induction in B10.S and B10 mice. Mice from both strains were immunized with doses of HBcAg, PC-HBeAg, P16, and either 120-131 or 129-140 known to elicit optimal T-cell proliferative responses, splenic T cells were cultured in vitro with the same Ag used for immunization, and cytokine production was measured in culture SN. As shown in Table 3, the dominant Th₁-like response observed in B10.S mice immunized with HBc/eAg particles was also apparent when B10.S mice were immunized with these alternative forms of the Ag. In general, IL-2 and IFN- γ production predominated the polyclonal T-cell responses regardless of the form of the immunogen. Note also the complete absence of IL-4 production after immunization with P16 and peptide 120-131. This may explain the inability of 120-131-primed F₁ (+/+) T cells to transfer autoantibody production in HBeAg-Tg F₁ recipients

FIG. 4. Comparison of HBc/eAg-specific cytokine production in PLN cell and splenic T-cell populations. Groups of five B10 mice were immunized with 10 µg of HBc/eAg in incomplete Freund's adjuvant in the hind footpads (A) or i.p. (B). Ten days after immunization, draining PLN cells (A) or spleen cells (B) were harvested, pooled, and cultured (8×10^6) in vitro with the indicated concentrations of HBc/eAg or peptide 129-140. Culture SN were collected at 48 h for IL-4 and IFN- γ determination. IL-4 production is expressed as proliferation of the IL-4-sensitive CT.4S cell line incubated with SN corrected for background proliferation elicited by SN from the cultures without Ag (Δ cpm). IFN- γ was measured by two-site ELISA and expressed as an optical density at 492 nm.

(Table 2). The effects of Ag structure on cytokine production in the B10 strain were not as straightforward as in the B10.S strain. Similar to immunization with HBc/eAg, immunization of B10 mice with HBcAg particles primed splenic T cells which produced IL-4 more efficiently than IFN- γ ; however, immunization with PC-HBeAg elicited weak IL-4 production which appeared less efficient than IL-2 and IFN- γ production (Table 3). The P16 Ag was a very poor immunogen in B10 mice and elicited no IL-4 production and low levels of IL-2 and IFN- γ production. The inability of P16 to prime for IL-4 production even in B10 mice cannot be explained by virtue of the monomeric nature and small size of P16 because immunization with the 12-residue peptide 129-140 primed T cells capable of producing IL-4 as well as IL-2 and IFN- γ . Furthermore, when P16 was used as an in vitro Ag and cultured with HBc/eAg-primed spleen cells (as in Table 2), it was unable to elicit IL-4 production from either B10.S or B10 splenic T cells (data not shown). Therefore, it appears the structure of the immunogen can influence the profile of cytokine production by HBc/eAg-specific Th cells.

TABLE 3. Effects of immunogen structure on cytokine production by B10.S and B10 splenic T cells^a

Strain	Immunogen	1/minimum Ag concn		
		IL-2	IL-4	IFN- γ
B10.S	HBcAg (5 µg)	625	5.0	100
	PC-HBeAg (10 µg)	12.5	0.5	0.5
	P16 (20 µg)	12.5	0	25
	120-131 (50 µg)	62.5	0	0.5
B10	HBcAg (5 µg)	62.5	125	25
	PC-HBeAg (10 µg)	2.5	0.5	2.5
	P16 (20 µg)	0.5	0	0.1
	129-140 (50 µg)	62.5	2.5	0.1

^a Groups of three mice each were immunized i.p. with the indicated amounts of HBcAg, PC-HBeAg, P16, or peptide 120-131 (B10.S) or 129-140 (B10) emulsified in incomplete Freund's adjuvant. Ten days later, spleen cells were harvested, pooled, and cultured (8×10^6 /ml) in vitro with various concentrations of the same Ag used for immunization, and culture SN were collected for IL-2, IL-4, and IFN- γ determination. The data are expressed as the reciprocal of the minimum Ag concentration required to elicit measurable amounts of the various cytokines.

DISCUSSION

The purpose of this study was to examine whether T-cell recognition of the HBV nucleocapsid Ags (i.e., HBcAg and HBeAg) was skewed toward either a Th₁- or Th₂-like response. The answer to this question depends largely on the MHC genotype of the responding murine strain. For example, B10.S (*H-2^s*) mice immunized with HBc/eAg particles exhibit a strong T-cell proliferative response and a weak T-cell helper function, and B10.S splenic HBc/eAg-specific T cells produce IL-2 and IFN- γ more efficiently than IL-4. This pattern is characteristic of a T-cell response dominated by Th₁-like cells. Conversely, B10 (*H-2^b*) mice immunized with HBc/eAg particles demonstrate relatively weak T-cell proliferative responses and strong Th-cell function, and in B10 mice, HBc/eAg-specific splenic T cells produce IL-4 more efficiently than IL-2 and IFN- γ . This pattern is characteristic of a T-cell response dominated by Th₂-like cells. Furthermore, the preferential Th₁ and Th₂ response patterns were reproduced whether native HBc/eAg or 12-residue synthetic T-cell sites were used as the immunizing Ags. Therefore, it appears that the combination of the T-cell site recognized and the MHC restricting element can in large part determine the Th phenotype of the HBc/eAg-specific T-cell response. The influence of T-cell epitope-MHC combination on Th₁-Th₂ phenotype has been previously described in several different systems (24, 41, 58). However, it is important to note that priming B10.S or B10 mice with HBc/eAg did not induce an exclusive Th₁ or Th₂ response, respectively, but rather elicited a mixed Th₁-Th₂ response which was dominated by either Th₁-like cells in B10.S mice or Th₂-like cells in B10 mice. The nonexclusivity of Th₁-Th₂ phenotype induction in this system is important in terms of examining approaches designed to modulate the balance between Th₁ and Th₂ cells.

In addition to T-cell epitope-MHC combination, other factors can also influence the Th phenotype of the HBc/eAg-specific T-cell response. For example, the inability of 120-131-primed, IA^s-restricted T cells to transfer T-cell help for anti-HBe antibody production in HBeAg-Tg mice was corrected by adding exogenous IL-4 to the *in vitro* culture prior to T-cell transfer. Similarly, reversal of the ability of 129-140-primed, IA^b-restricted T cells to transfer anti-HBe antibody production in this Tg model can be achieved by adding exogenous IL-12 to the culture (unpublished data). Therefore, as noted in other *in vitro* and *in vivo* systems (14, 19, 23, 46, 47, 50, 55, 59), the cytokine milieu is an important factor in determining the predominant Th phenotype of the developing T-cell response to HBc/eAg. In this regard, priming of HBc/eAg-specific Th subsets in the context of an HBV infection may differ from what is observed after immunization. We are currently examining the possibility of preferential Th-cell subset induction to HBc/eAg, using a variety of methods to elicit T-cell activation which may more accurately mimic a viral infection (i.e., vaccinia virus vectors and plasmid DNA).

We also observed a compartmentalization of HBc/eAg-specific Th₁ and Th₂ cells. In HBc/eAg-immunized B10 mice, T cells derived from the peripheral draining lymph node preferentially produced IFN- γ , whereas splenic T cells preferentially produced IL-4 upon stimulation with HBc/eAg or peptide 129-140 *in vitro*. This may be an important factor in the context of an infection because HBV is an hepatotropic virus, and priming and/or migration of Th cells in the liver may vary between the subsets. Other investigators have reported a unique tissue distribution for Th₁ and Th₂ cells; however, the distribution pattern appears to be Ag specific because the spleen has been reported to be the site of Th₁ as well as Th₂ migration (4, 21, 22, 45). Lastly, the context of the 129-140 T-cell epitope within

various structural forms influenced the Th₁-Th₂ profile to some degree. For example, B10.S mice immunized with HBc/eAg particles demonstrate a predominant Th₁ response, but Th₂ activity (i.e., IL-4 production) is also observable. However, B10.S mice immunized with P16 or peptide 120-131 generate an exclusive Th₁-like response. Similarly, immunization with P16 failed to elicit IL-4 production in B10 mice, which demonstrate a bias toward the Th₂ phenotype when immunized with HBc/eAg particles. These observations may have practical implications in terms of suggesting a method to manipulate the predominant Th phenotype simply by choosing the appropriate structural form of the Ag to include in a potential therapeutic vaccine.

Historically, a number of factors have been described which may influence whether an immune response will be dominated by Th₁ or Th₂ cells, including the type of Ag-presenting cells (APC) (5, 62), the Ag dose (2, 42), the adjuvant used (15); immunization route and protocol (4), tissue compartment (4, 21, 22, 45), local cytokine environment (14, 19, 23, 46, 47, 50, 55), and epitope specificity (24, 41, 58). Several recent studies have demonstrated that monoclonal naive T cells (i.e., from TCR-Tg mice) can differentiate along either the Th₁ or Th₂ pathway dependent on the cytokine present in the priming culture. It appears that IL-4 is essential for development of Th₂ cells and that IL-12 enhances the development of Th₁ cells (18, 19, 47, 54, 55). These studies suggest that the cytokines present in the priming environment are the major factor responsible for determining the Th phenotype of developing T cells. These studies also indicate that Th₁ and Th₂ cells do not represent precommitted and separate cell lineages prior to exposure to Ag but rather are a product of a differentiation step associated with Ag-specific activation (43).

If Th₁ and Th₂ cells do not possess separate TCR repertoires, how then can epitope specificity influence Th phenotype selection? Earlier studies in a neonatal tolerance system defined another significant difference between the 120-131 and the 129-140 T-cell epitopes of HBc/eAg in addition to the propensity to elicit different Th subsets. Neonatal injection of B10.S mice with 120-131 induced efficient T-cell tolerance specific for the peptide as well as for native HBc/eAg, whereas neonatal injection of B10 mice with 129-140 failed to elicit T-cell tolerance (32). Furthermore, the immunogenic and tolerogenic peptide 120-131 was converted into an immunogenic and nontolerogenic peptide, similar to 129-140, by a single amino acid substitution for an agretropic residue. This study demonstrated that peptide-MHC affinity could influence T-cell tolerance induction. It was argued that 129-140 bound MHC class II molecules with lower affinity than 120-131, which would result in a lower peptide-MHC ligand density on the APC in the thymus and allow greater numbers of 129-140-specific T cells to evade negative selection (32). Interestingly, mice immunized with human collagen IV develop either antibody responses or proliferative T-cell responses as a function of MHC genotype similar to the HBc/eAg system described herein (41, 60). In the collagen system, it has been suggested that because different or the same peptide T-cell site may bind different MHC class II molecules with different efficiencies, the peptide-MHC ligand density on the APC may determine the differentiation of Th₁ or Th₂ cells by virtue of the strength of the TCR-mediated signal (41). Therefore, the tendency to differentiate along the Th₂ pathway and the ability to escape negative selection in the thymus may both be related to low-affinity peptide-MHC interactions. Indeed, Th₂ cells have been reported to be less susceptible to tolerance induction than Th₁ cells (3, 8, 49). In the HBc/eAg system, the 129-140-specific T-cell proliferative response is significantly more sensitive than

the 120-131-specific T-cell response to inhibition with an anti-CD4 MAb (data not shown), which is consistent with the reduced avidity of 129-140-specific T-cell recognition. Possibly, low-avidity T-cell recognition of the 129-140 T-cell site on HBeAg in the context of IA^b preferentially elicits a Th₂-like response, whereas high-avidity T-cell recognition of the 120-131 site in the context of IA^s elicits a predominant Th₁-like response. In (B10 × B10.S)F₁ mice, one may expect to observe cross-regulation between 129-140- and 120-131-specific Th cells by virtue of the mutually inhibitory cytokines produced by Th₂ and Th₁ cells. Indeed, in preliminary experiments, we have obtained evidence that 120-131-specific F₁ T cells can down regulate 129-140-specific Th-cell function (unpublished observation). Therefore, in an individual heterozygous within the MHC, it may be difficult to discern dominant Th subsets. Furthermore, the Th₁-Th₂ balance is, of course, a more complex situation in the context of an HBV infection. During an infection, there are multiple viral proteins each expressing multiple T-cell epitopes. If each T-cell epitope-MHC combination elicits a biased Th₁ or Th₂ response, one would expect at least a mixture of Th₁- and Th₂-dominated responses. It is also possible that viral infections in general are dominated by Th₁-biased responses due to endogenous interferon production. Chronic HBV infection may be unique in this respect because of the possible role HBeAg-specific Th-cell tolerance plays in the induction and maintenance of chronic HBV infection. For example, in HBeAg-Tg mice, the presence of HBeAg preferentially eliminates the Th₁ subset over the Th₂ subset (27a, 30). A similar skewing of HBeAg-specific T cells toward the Th₂ subset may occur in neonates infected with HBV.

In summary, it appears that many factors can influence the differentiation into a Th₁ or Th₂ cell. However, these factors need not be mutually exclusive. A dominant influence may well be the local cytokine environment at the time the naive T-cell precursor first recognizes the immunogen. However, the impact of epitope specificity on Th phenotype development should not be underestimated. In the HBeAg system, two separate 12-residue Th-cell sites injected identically with the same adjuvant (CFA or incomplete Freund's adjuvant) into the same F₁ mouse resulted in distinct Th subset priming. Presumably, the local nonspecific cytokine environment, the APC, route of immunization, etc., were identical regardless of which peptide was injected. In this situation, factors inherent to the TCR-peptide-MHC interaction appear to direct Th-cell phenotype differentiation. Subsequently, Ag-specific lymphokine production may regulate the Th₁-Th₂ cell balance.

The demonstration that the nucleocapsid Ags of HBV can be recognized preferentially by Th₁ or Th₂ cells may have clinical relevance. Previous studies suggested that the different serological and biochemical profiles of patients with acute, symptomatic chronic and asymptomatic chronic HBV infections may reflect differences in HBeAg-specific Th phenotype (26, 27). Noncytopathic viral infections including HBV possess intracellular and extracellular components. Efficient viral clearance requires an adequate cellular immune response to eliminate infected cells and an adequate humoral response to prevent the spread of free virus to adjacent cells. Therefore, the balance between HBV-specific Th₁ and Th₂-like responses may determine the resolution of an adult onset of acute HBV infection or the degree of liver injury in a chronic infection. Additionally, the greatest number of chronic HBV infections are attributable to vertical transmission. We have proposed that HBeAg-specific T-cell tolerance initiated in utero accounts for the inability of the infant to clear a perinatal or postnatal HBV infection (30, 31). It is interesting that in an HBeAg-Tg model of chronic HBV infection, the HBeAg-spe-

cific T cells that preferentially evade tolerance induction and survive in the periphery are 129-140 specific and predominantly of the Th₂ subset (27a, 30, 37). Similarly, the residual HBeAg-specific T cells present in postnatally infected HBV carriers may be skewed toward the Th₂ subset. In this event, a goal of immunomodulatory therapy in patients with chronic HBV infection may be to shift the balance of HBeAg-specific T cells from Th₂ to Th₁ predominance.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants AI20720, AI33562, and AI15955, and the WHO/UNDP program for Vaccine Development.

REFERENCES

1. Belosevic, M., D. S. Finbloom, P. van der Meide, M. Z. Slayter, and C. A. Nacy. 1989. Administration of monoclonal anti-IFN- γ antibodies *in vivo* abrogates natural resistance of C3H/HeN mice to infection with *Leishmania major*. *J. Immunol.* **143**:266-274.
2. Bretscher, P. A., G. Wei, J. N. Menon, and H. Bielefeldt-Olmann. 1992. Establishment of stable, cell-mediated immunity that makes "susceptible" mice resistant to *Leishmania major*. *Science* **257**:539-542.
3. Burstein, H. J., C. M. Shea, and A. K. Abbas. 1992. Aqueous antigens induce *in vivo* tolerance selectively in IL-2- and IFN- γ -producing (Th1) cells. *J. Immunol.* **148**:3687-3691.
4. Caulada-Benedetti, Z., F. Al-Zamel, A. Sher, and S. James. 1991. Comparison of Th1- and Th2-associated immune reactivities stimulated by single versus multiple vaccination of mice with irradiated *Schistosoma mansoni* cercariae. *J. Immunol.* **146**:1655-1660.
5. Chang, T.-L., C. M. Shea, S. Urioste, R. C. Thompson, W. H. Boom, and A. K. Abbas. 1990. Heterogeneity of helper/inducer T lymphocytes. III. Responses of IL-2- and IL-4-producing (Th1 and Th2) clones to antigens presented by different accessory cells. *J. Immunol.* **145**:2803-2812.
6. Chatelain, R., K. Varkila, and R. L. Coffman. 1992. IL-4 induces a Th2 response in *Leishmania major*-infected mice. *J. Immunol.* **148**:1182-1187.
7. D'Andreas, A., M. Rengaraju, N. M. Valiante, J. Chemhimi, M. Kubin, M. Aste, S. H. Chan, M. Kobayashi, D. Young, and E. Nickbarg. 1992. Production of natural killer cell stimulatory factor (NKSF) by peripheral blood mononuclear cells. *J. Exp. Med.* **176**:1387-1398.
8. De Wit, D., M. Van Mechelen, M. Ryelandt, A. C. Figueiredo, D. Abramowicz, M. Goldman, H. Bazin, J. Urbain, and O. Leo. 1992. The injection of deaggregated gamma globulins in adult mice induces antigen-specific unresponsiveness of T helper type 1 but not type 2 lymphocytes. *J. Exp. Med.* **175**:9-14.
9. Fernandez-Botran, R., V. M. Sanders, T. R. Mosmann, and E. S. Vitetta. 1988. Lymphokine-mediated regulation of the proliferative response of clones of T helper 1 and T helper 2 cells. *J. Exp. Med.* **168**:543-551.
10. Ferrari, C., A. Penna, A. Bertoletti, A. Valli, A. D. Antoni, T. Giuberti, A. Cavalli, M.-A. Petit, and F. Fiaccadori. 1990. Cellular immune response to hepatitis B virus-encoded antigens in acute and chronic hepatitis B virus infection. *J. Immunol.* **145**:3442-3449.
11. Finkelman, F. D., J. Holmes, I. M. Katona, J. F. Urban, Jr., M. P. Beckmann, L. S. Park, K. A. Schooley, R. L. Coffman, T. R. Mosmann, and W. E. Paul. 1990. Lymphokine control of *in vivo* immunoglobulin isotype selection. *Annu. Rev. Immunol.* **8**:303-333.
12. Fiorentino, D. F., M. W. Bond, and T. R. Mosmann. 1989. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J. Exp. Med.* **170**:2081-2092.
13. Gajewski, T. F., and F. W. Fitch. 1988. Anti-proliferative effect of IFN- γ in immune regulation. I. IFN- γ inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. *J. Immunol.* **140**:4245-4253.
14. Gross, A., S. Z. Ben-Sasson, and W. E. Paul. 1993. Anti-IL-4 diminishes *in vivo* priming for antigen-specific IL-4 production by T cells. *J. Immunol.* **150**:2112-2119.
15. Grun, J. L., and P. H. Maurer. 1989. Different T helper cell subsets elicited in mice utilizing two different adjuvant vehicles: the role of endogenous interleukin 1 in proliferative responses. *Cell. Immunol.* **121**:134-145.
16. Haak-Frednscho, M., J. F. Brown, Y. Itzawa, R. D. Wagner, and C. J. Czuprynski. 1992. Administration of anti-IL-4 monoclonal antibody 11B11 increases the resistance of mice to *Listeria monocytogenes* infection. *J. Immunol.* **148**:3978-3985.
17. Houghten, R. A. 1985. General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids. *Proc. Natl. Acad. Sci. USA* **82**:5131-5135.
18. Hsieh, C. S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra, and K. M. Murphy. 1993. Development of Th1 CD4⁺ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science* **260**:547-549.
19. Hsieh, D. S., A. B. Heimberger, J. S. Gold, A. O'Garra, and K. M. Murphy.

1992. Differential regulation of T helper phenotype development by interleukins 4 and 10 in an alpha beta T cell-receptor transgenic system. *Proc. Natl. Acad. Sci. USA* **89**:6065–6069.
20. Hu-Li, J., J. Ohara, C. Watson, W. Tsang, and W. E. Paul. 1989. Derivation of a T cell line that is highly responsive to IL-4 and IL-2 (CT.4R) and of an IL-2 hyporesponsive mutant of that line (CT.4S). *J. Immunol.* **142**:800–807.
21. Kelly, E. A. B., E. S. Cruz, K. M. Hauda, and D. L. Wassom. 1991. IFN- γ and IL-5-producing cells compartmentalize to different lymphoid organs in *Trichinella spiralis*-infected mice. *J. Immunol.* **147**:306–317.
22. Kullberg, M. C., E. J. Pearce, S. E. Hieny, A. Sher, and J. A. Berzofsky. 1992. Infection with *Schistosoma mansoni* alters Th1/Th2 cytokine responses to a non-parasite antigen. *J. Immunol.* **148**:3264–3270.
23. Le Gros, G., S. Z. Ben-Sasson, R. Seder, F. D. Finkelman, and W. E. Paul. 1990. Generation of interleukin-4 (IL-4)-producing cells *in vivo* and *in vitro*: IL-2 and IL-4 are required for *in vitro* generation of IL-4-producing cells. *J. Exp. Med.* **172**:921–931.
24. Liew, F. Y., S. M. Millott, and J. A. Schmidt. 1990. A repetitive peptide of *Leishmania* can activate T helper type 2 cells and enhance disease progression. *J. Exp. Med.* **172**:1359–1365.
25. Locksley, R. M., and P. Scott. 1991. Helper T-cell subsets in mouse leishmaniasis: induction, expansion and effector function. *Immunol. Today* **12**:A58–A61.
26. Maruyama, T., A. McLachlan, S. Iino, K. Koike, K. Kurokawa, and D. R. Milich. 1993. The serology of chronic hepatitis B infection revisited. *J. Clin. Invest.* **91**:2586–2585.
27. Maruyama, T., F. Schödel, S. Iino, K. Koike, K. Yasuda, D. Peterson, and D. R. Milich. 1994. Distinguishing between acute and symptomatic chronic hepatitis B virus infection. *Gastroenterology* **106**:1006–1015.
- 27a. Milich, D. R., et al. Submitted for publication.
28. Milich, D. R., J. L. Hughes, R. Houghten, A. McLachlan, and J. E. Jones. 1989. Functional identification of agretopic and epitopic residues within an HBcAg T cell determinant. *J. Immunol.* **143**:3141–3147.
29. Milich, D. R., J. L. Hughes, A. McLachlan, G. B. Thornton, and A. Moriarty. 1988. Hepatitis B synthetic immunogen comprised of nucleocapsid T-cell sites and an envelope B-cell epitope. *Proc. Natl. Acad. Sci. USA* **85**:1610–1614.
30. Milich, D. R., J. Jones, J. Hughes, and T. Maruyama. 1993. Role of T-cell tolerance in the persistence of hepatitis B virus infection. *J. Immunotherapy* **14**:226–223.
31. Milich, D. R., J. E. Jones, J. L. Hughes, J. Price, A. K. Raney, and A. McLachlan. 1990. Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance *in utero*? *Proc. Natl. Acad. Sci. USA* **87**:6599.
32. Milich, D. R., J. E. Jones, A. McLachlan, R. Houghten, G. B. Thornton, and J. L. Hughes. 1989. Distinction between immunogenicity and tolerogenicity among HBcAg T cell determinants: influence of peptide-MHC interaction. *J. Immunol.* **143**:3148–3156.
33. Milich, D. R., P. S. Linsley, J. L. Hughes, and J. E. Jones. 1994. Soluble CTLA-4 can suppress autoantibody production and elicit long term unresponsiveness in a novel transgenic model. *J. Immunol.* **153**:429–435.
34. Milich, D. R., and A. McLachlan. 1986. The nucleocapsid of hepatitis B virus is both a T-cell-independent and a T-cell-dependent antigen. *Science* **234**:1398–1401.
35. Milich, D. R., A. McLachlan, F. V. Chisari, and G. B. Thornton. 1986. Nonoverlapping T and B cell determinants on a hepatitis B surface antigen pre-S(2) region synthetic peptide. *J. Exp. Med.* **164**:532–541.
36. Milich, D. R., A. McLachlan, A. Moriarty, and G. B. Thornton. 1987. Immune response to hepatitis B virus core antigen (HBcAg): localization of T cell recognition sites within HBcAg/HBeAg. *J. Immunol.* **139**:1223–1231.
37. Milich, D. R., A. McLachlan, A. K. Raney, R. Houghten, G. B. Thornton, T. Maruyama, J. L. Hughes, and J. E. Jones. 1991. Autoantibody production in hepatitis B e antigen transgenic mice elicited with a self T-cell peptide and inhibited with nonself peptides. *Proc. Natl. Acad. Sci. USA* **88**:4348–4352.
38. Milich, D. R., A. McLachlan, S. Stahl, P. Wingfield, G. B. Thornton, J. L. Hughes, and J. E. Jones. 1988. Comparative immunogenicity of hepatitis B virus core and e antigens. *J. Immunol.* **141**:3617–3624.
39. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* **136**:2348–2357.
40. Mosmann, T. R., and T. Fong. 1989. Specific assays for cytokine production by T cells. *J. Immunol. Methods* **116**:151–159.
41. Murray, J. S., C. Pfeiffer, J. Madri, and K. Bottomly. 1992. Major histocompatibility complex (MHC) control of CD4 T cell subset activation. II. A single peptide induces either humoral or cell-mediated responses in mice of distinct MHC genotype. *Eur. J. Immunol.* **22**:559–565.
42. Parish, C. R., and F. Y. Liew. 1972. Immune response to chemically modified flagellin. III. Enhanced cell-mediated immunity during high and low zone antibody tolerance to flagellin. *J. Exp. Med.* **135**:298–310.
43. Paul, W. E., and R. A. Seder. 1994. Lymphocyte responses and cytokines. *Cell* **76**:241–251.
44. Pearce, E. J., P. Caspar, J.-M. Grzych, F. A. Lewis, and A. Sher. 1991. Downregulation of Th1 cytokine production accompanies induction of Th2 responses by a parasitic helminth, *Schistosoma mansoni*. *J. Exp. Med.* **173**:159–167.
45. Pond, L., D. L. Wassom, and C. E. Hayes. 1992. Influence of resistant and susceptible genotype, IL-1, and lymphoid organ on *Trichinella spiralis*-induced cytokine secretion. *J. Immunol.* **149**:957–965.
46. Reiner, S. L., Z.-E. Wang, F. Hatam, P. Scott, and R. M. Locksley. 1993. T_H1 and T_H2 cell antigen receptors in experimental leishmaniasis. *Science* **259**:1457–1460.
47. Röcken, M., J. H. Saurat, and C. Hauser. 1992. A common precursor for CD4⁺ T cells producing IL-2 or IL-4. *J. Immunol.* **148**:1031–1036.
48. Romani, L., A. Mencacci, U. Grohmann, S. Mocchi, P. Mosci, P. Puccetti, and F. Bistoni. 1992. Neutralizing antibody to interleukin 4 induces systemic protection and T helper type 1-associated immunity in murine candidiasis. *J. Exp. Med.* **176**:19–25.
49. Romball, C. G., and W. O. Weigle. 1993. *In vivo* induction of tolerance in murine CD4⁺ cell subsets. *J. Exp. Med.* **178**:1637–1644.
50. Sadick, M. D., F. P. Heinzel, B. J. Holaday, R. T. Pu, R. S. Dawkins, and R. M. Locksley. 1990. Cure of murine leishmaniasis with anti-interleukin-4 monoclonal antibody. Evidence for a T cell-dependent, interferon gamma-independent mechanism. *J. Exp. Med.* **171**:115–122.
51. Salgame, P., J. S. Abrama, C. Clayberger, H. Goldstein, J. Convit, R. L. Modlin, and B. R. Bloom. 1991. Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. *Science* **254**:279–282.
52. Schödel, F., A. M. Moriarty, D. L. Peterson, J. Zheng, J. L. Hughes, H. Will, D. J. Leturcq, J. S. McGee, and D. R. Milich. 1992. The position of heterologous epitopes inserted in hepatitis B virus core particles determines their immunogenicity. *J. Virol.* **66**:106–114.
53. Schödel, F., D. Peterson, J. Zheng, J. E. Jones, J. L. Hughes, and D. R. Milich. 1993. Structure of hepatitis B virus core and e-antigen: a single precore amino acid prevents nucleocapsid assembly. *J. Biol. Chem.* **268**:1332–1337.
54. Seder, R. A., R. Gazzinelli, A. Sher, and W. E. Paul. 1993. IL-12 acts directly on CD4⁺ T cells to enhance priming for IFN γ production and diminishes IL-4 inhibition of such priming. *Proc. Natl. Acad. Sci. USA* **90**:10188–10192.
55. Seder, R. A., W. E. Paul, M. M. Davis, and B. Fazekas de St. Groth. 1992. The presence of interleukin-4 during *in vitro* priming determines the lymphokine-producing potential of CD4⁺ T cells from T cell receptor transgenic mice. *J. Exp. Med.* **176**:1091–1098.
56. Schaeffer, E. B., A. Sette, D. L. Johnson, M. C. Bekoff, J. A. Smith, H. M. Grey, and S. Buss. 1989. Relative contribution of “determinant selection” and “holes in the T cell repertoire” to T cell responses. *Proc. Natl. Acad. Sci. USA* **86**:4649–4653.
57. Sher, A., and R. L. Coffman. 1992. Regulation of immunity to parasites by T cells and T cell-derived cytokines. *Annu. Rev. Immunol.* **10**:385–409.
58. Soloway, P., S. Fish, H. Passmore, M. Geffer, R. Coffee, and T. Manser. 1991. Regulation of the immune response to peptide antigens: differential induction of immediate-type hypersensitivity and T cell proliferation due to changes in either peptide structure or major histocompatibility complex haplotype. *J. Exp. Med.* **174**:847–858.
59. Swain, S. L., A. D. Weinberg, M. English, and G. Huston. 1990. IL-4 directs the development of Th2-like helper effectors. *J. Immunol.* **145**:3796–4004.
60. Tite, J. P., H. G. Foellmer, J. A. Madri, and C. A. Janeway, Jr. 1987. Inverse Ir gene control of the antibody and T cell proliferative responses to human basement membrane collagen. *J. Immunol.* **139**:2892–2898.
61. Urban, J. J., I. M. Katona, W. E. Paul, and F. D. Finkelman. 1991. Interleukin-4 is important in protective immunity to a gastrointestinal nematode infection in mice. *Proc. Natl. Acad. Sci. USA* **88**:5513–5517.
62. Weaver, C. T., C. M. Hawrylowicz, and E. R. Unanue. 1988. T helper cell subsets require the expression of distinct costimulatory signals by antigen-presenting cells. *Proc. Natl. Acad. Sci. USA* **85**:8181–8185.