Aberrant Lymphocyte Activation Precedes Delayed Virus-Specific T-Cell Response after both Primary Infection and Secondary Exposure to Hepadnavirus in the Woodchuck Model of Hepatitis B Virus Infection[▽]

Shashi A. Gujar,¹ Adam K. Jenkins,¹ Clifford S. Guy,¹ Jinguo Wang,¹ and Tomasz I. Michalak^{1,2*}

Molecular Virology and Hepatology Research Group, Division of BioMedical Sciences,¹ and Discipline of Laboratory Medicine,² Faculty of Medicine, Health Sciences Centre, Memorial University, St. John's, Newfoundland A1B 3V6, Canada

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The contribution of virus-specific T lymphocytes to the outcome of acute hepadnaviral hepatitis is well recognized, but a reason behind the consistent postponement of this response remains unknown. Also, the characteristics of T-cell reactivity following reexposure to hepadnavirus are not thoroughly recognized. To investigate these issues, healthy woodchucks (*Marmota monax*) were infected with liver-pathogenic doses of woodchuck hepatitis virus (WHV) and investigated unchallenged or after challenge with the same virus. As expected, the WHV-specific T-cell response appeared late, 6 to 7 weeks postinfection, remained high during acute disease, and then declined but remained detectable long after the resolution of hepatitis. Interestingly, almost immediately after infection, lymphocytes acquired a heightened capacity to proliferate in response to mitogenic (nonspecific) stimuli. This reactivity subsided before the WHV-specific T-cell response appeared, and its decline coincided with the cells' augmented susceptibility to activation-induced death. The analysis of cytokine expression profiles confirmed early *in vivo* activation of immune cells and revealed their impairment of transcription of tumor necrosis factor alpha and gamma interferon. Strikingly, reexposure of the immune animals to WHV swiftly induced hyperresponsiveness to nonspecific stimuli, followed again by the delayed virus-specific response. Our data show that both primary and secondary exposures to hepadnavirus induce aberrant activation of lymphocytes preceding the virus-specific T-cell response. They suggest that this activation and the augmented death of the cells activated, accompanied by a defective expression of cytokines pivotal for effective T-cell priming, postpone the adaptive T-cell response. These impairments likely hamper the initial recognition and clearance of hepadnavirus, permitting its dissemination in the early phase of infection.

Hepatitis B virus (HBV) causes acute liver inflammation which may advance to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (7). In the majority of adults, the immune responses induced by HBV are sufficient to resolve acute hepatitis (AH), although they commonly fail to eliminate the virus entirely, leading to an indefinitely long persistence of trace levels of virus replication in the liver and cells of the immune system (43, 45, 51, 52, 66). This residual virus carriage, currently termed secondary occult HBV infection (41, 44), is accompanied by protracted HBV-specific T-cell proliferative and cytotoxic T-lymphocyte (CTL) responses (22, 52, 60), persistently elevated intrahepatic transcription of gamma interferon (IFN- γ) and tumor necrosis factor alpha (TNF- α), and residual liver inflammation in woodchucks convalescent from AH (24, 42). The contributions of the innate and adaptive immunities to the control of HBV infection have been well delineated overall (22). Among other findings, it was established that the recovery from AH is accompanied by robust polyclonal and multispecific HBV-specific T-helper type 1 (Th1)

proliferative and CTL responses (15, 21). These virus-specific reactivities were found to be preceded by upregulated hepatic expression of IFN- γ and TNF- α , suggesting activation of the local innate immunity (20, 60). However, contrary to responses to infections with other viruses, HBV is seemingly ignored by the CD4⁺ and CD8⁺ T cells for about 2 months after primary infection, even when the infection is finally resolved (4, 63). This delay is accompanied by a very low frequency of HBV-specific CTL, which usually does not exceed 1% of circulating CD8⁺ T cells during the acute phase of self-limiting infection (31). This contrasts with the responses to infections with other viral pathogens where the primary adaptive T-cell responses can be detected as early as 5 to 10 days postinfection and in which up to 10% of the peripheral CD8⁺ T cells might consist of virus-specific CTL (29, 54, 64). A reason behind this delay in the appearance and the relative weakness of the adaptive T-cell response in hepadnaviral infection is unknown. Nonetheless, it is likely that this situation may hinder the initial control of viremia and the complete clearance of hepadnavirus, as evidenced by the common occurrence of low-level (occult) infection persisting after the resolution of AH (43, 45, 51). Furthermore, it is generally accepted that recovery from acute hepatitis B is associated with complete protection from a reoccurrence of HBV infection. However, the nature and characteristics of the cellular immune responses following reexposure to hepadnavirus are not defined, and a primary protective

* Corresponding author. Mailing address: Molecular Virology and Hepatology Research Group, Division of BioMedical Science, Faculty of Medicine, Health Sciences Centre, Memorial University, St. John's, NL A1B 3V6, Canada. Phone: (709) 777-7301. Fax: (709) 777-8279. E-mail: timich@mun.ca.

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role for anti-HBV antibodies in this regard is acknowledged (5, 53, 56, 67).

The infection of woodchucks (*Marmota monax*) with woodchuck hepatitis virus (WHV) represents a highly valuable natural model of HBV infection in which the sequella of virological and molecular events and the patterns of virus-induced liver disease closely resemble those occurring in humans (36, 37, 39). A significant degree of antigenic cross-reactivity between HBV and WHV and similarities in the profiles of immune responses induced by both infections make investigations in the woodchuck-WHV model highly relevant to the recognition of immunological events occurring after the primary exposure and subsequent reexposures to HBV.

For the present study, we dissected the profiles of hepadnavirus-specific and generalized (nonspecific, mitogen-induced) proliferative T-cell responses during the preacute and acute phases of WHV infection and after challenge and rechallenge with the same virus, along with the expression of cytokines affiliated with immune-cell activation. We also investigated the susceptibility of lymphoid cells to apoptotic death during the progress of acute infection. We have found that lymphocytes after primary and secondary exposures to WHV acquire almost immediately a strongly augmented capacity to proliferate in response to virus-nonspecific (mitogenic) stimuli, which each time precedes the delayed appearance of the virus-specific T-cell response. This *ex vivo* evident hyperreactivity coincides with the augmented expression of cytokines in unmanipulated, circulating lymphoid cells, indicative of their *in vivo* activation, and with an inhibited expression of TNF- α and IFN- γ , suggesting their impaired function. These findings provide new insights into the properties of T-cell responses accompanying the early stages of hepadnaviral infection and following reexposure to pathogenic hepadnavirus. They raise a possibility that a strong aberrant activation of lymphocytes occurring immediately after exposure to hepadnavirus contributes to the postponement of virus-specific primary, as well as secondary, T-cell responses. These events may hinder the initial recognition and elimination of the virus, facilitating its dissemination in the prodromal phase of infection.

MATERIALS AND METHODS

Animals and WHV inoculations. Ten healthy, adult woodchucks housed in the Woodchuck Hepatitis Research Facility at Memorial University, St. John's, Canada were infected with large, liver-pathogenic doses of WHV. Prior exposure to WHV was excluded based on negative serological markers for WHV infection and the absence of WHV DNA in randomly selected serum, peripheral blood mononuclear cell (PBMC), and liver biopsy samples assessed by nested PCR-nucleic acid hybridization (PCR-NAH) assays (sensitivity, <10 virus genome equivalents [vge]/ml) (9, 42). The animals were divided into three groups. Four animals (group A) were investigated in the first phase to determine the overall features of T-cell proliferative responses accompanying primary infection and subsequent exposures to hepadnavirus. The animals were intravenously injected with 1.9×10^{11} DNase-protected vge of WHV/tm4 inoculum carrying the wild-type WHV, as determined by a whole-genome sequence analysis (P. M. Mulrooney-Cousins and T. I. Michalak, unpublished data). They were followed for 65 weeks post-primary infection (w.p.p.i.) and then challenged with the same dose of WHV/tm4 and rechallenged 15 weeks later with either 1.9×10^{11} vge (animals 1/F and 2/F) or 1.9×10^2 vge (animals 3/F and 4/M) of the inoculum (Fig. 1A). Four other woodchucks constituted study group B. These animals were injected once with WHV, followed for 16 w.p.p.i., and investigated to investigate a possible interdependence between the heightened T-lymphocyte proliferation capacity occurring immediately after WHV exposure identified in the first study and the lymphocyte susceptibility to apoptotic death, the delayed WHV-specific

T-cell response, and the expression of selected proinflammatory and antiviral cytokines in lymphoid cells. In this group, two animals (5/M and 6/M) were infected with 1.9×10^{11} vge of WHV/tm4, while two others (7/M and 8/M) were infected with a WHV/tm3 inoculum (GenBank accession number AY334075) (41) at 1.1×10^{10} DNase-protected vge per dose (Fig. 1B). In addition, two woodchucks, each injected with a single dose of WHV/tm4 at 1.9×10^{11} vge, were examined for 112 weeks postinfection (w.p.i.) as controls. The study protocol was approved by the Institutional President's Committee on Animal Bioethics and Care.

Sample collection. Animals were bled at the time points indicated in Fig. 1. Sera were collected and preserved. PBMC were isolated as described before (23). Their viability normally exceeded 98%, as determined by trypan blue dye exclusion. Liver biopsies were obtained by surgical laparotomy at the time points showed in Fig. 1 and examined for WHV DNA load and histological lesions using the scoring criteria reported before (40–42).

Serological and WHV DNA detection assays. Serial serum samples were tested for WHV surface antigen (WHsAg), antibodies to WHV core antigen (anti-WHc), and antibodies to WHsAg (anti-WHs) by using in-house specific-enzyme-linked immunoassays reported before (9, 41, 42). The levels of WHV DNA in sera and liver biopsies were quantified by real-time PCR (41) and, when negative, by PCR-NAH assay, as reported previously (42).

WHV antigens and mitogens for T-cell proliferation assays. Recombinant WHV core, e, and X proteins, designated rWHc, rWHe, and rWHx, respectively, and used to measure WHV-specific T-cell proliferation *in vitro*, were produced in the pET41b(+) *Escherichia coli* expression system (Novagen, Darmstadt, Germany) and affinity purified as recently reported (62). They were extensively tested for nonspecific T-cell proliferation against potential bacterial contaminants and found to be entirely devoid of such activity (62). In addition, a synthetic WHV peptide, containing the immunodominant epitope corresponding to amino acids 97 to 110 (WHc_{97–110}) of the virus nucleocapsid protein (33), was synthesized. WHV envelope particles carrying WHsAg specificity were purified from pooled sera of a chronic WHV carrier, as described previously (62). As inducers of generalized, nonspecific, polyclonal lymphocyte responses, concanavalin A (ConA; Pharmacia Fine Chemicals, Uppsala, Sweden), pokeweed mitogen (PWM, *Phytolacca americana* agglutinin; ICN Biochemicals, Inc., Aurora, OH), and phytohemagglutinin (PHA; ICN Biochemicals) were used (see below).

Adenine incorporation T-cell proliferation assay. A [3 H]adenine incorporation T-lymphocyte proliferation assay was applied to examine WHV-specific and mitogen-induced T-cell responsiveness and performed as described elsewhere (23, 30). Briefly, freshly isolated PBMC were cultured in 96-well flat-bottomed plates (Becton Dickinson Labware, Franklin Lakes, NJ) at a density of 1×10^5 cells/well in complete AIM-V lymphocyte culture medium (Gibco-Invitrogen Corp., Auckland, New Zealand) with 10% fetal calf serum (Gibco-Invitrogen). Recombinant WHV proteins and WHsAg were added in triplicate at 1 μ g/ml and 2 μ g/ml, whereas the WHc_{97–110} peptide was used at five twofold dilutions from 1.25 μ g/ml to 20 μ g/ml. Similarly, to assess the generalized lymphocyte response, each PBMC sample was exposed in triplicate to five twofold dilutions of ConA or PHA from 1.25 μ g/ml to 20 μ g/ml and PWM from 0.6 μ g/ml to 10 μ g/ml or to medium alone as a control. The cultures were incubated for 96 h at 37°C, pulsed with 0.5 μ Ci of [3 H]adenine (Amersham Pharmacia Biotech, AB, Uppsala, Sweden) per well, and incubated for an additional 12 to 18 h. Then, the cells were harvested and the radioactivity counts per minute (cpm) in each well were determined (23). The mean cpm values for either stimulated or control cells were calculated by averaging the counts in the respective triplicate wells. The stimulation index (SI) was calculated by dividing the mean cpm obtained either after WHV antigen or mitogen stimulation by the mean cpm detected in control, unstimulated wells. SI values of ≥ 3.1 for rWHc, rWHe, and rWHx and ≥ 2.1 for WHc_{97–110} and WHsAg were considered as positive based on the cutoff values given by 3 standard deviations above the mean values obtained from control wells. The mean mitogenic SI (MMSI) was determined by averaging the SI values obtained for all five concentrations of a given mitogen, as described previously (23). For each time point, the data were presented as the MMSI, which was calculated by averaging the SI values obtained after stimulation with five different concentrations of a given mitogen.

CFSE flow cytometry T-cell proliferation assay. Since WHV-specific T-cell responses examined by using a [3 H]adenine incorporation assay gave readings at the detection limit of the assay in samples collected after the third injection with WHV, i.e., 80 w.p.p.i., and to confirm the observations made for study group A in group B animals, a more-sensitive flow cytometric assay with 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, Oregon) was employed as recently reported (23). Briefly, woodchuck PBMC were labeled with 1 μ M of CFSE and either stimulated with WHV antigens or

FIG. 1. General outline of the experimental protocols showing time points of injections with WHV and of collection of serum, PBMC, and liver biopsy samples; serological markers of WHV infection and serum WHV DNA detected; hepatic WHV loads; and the results of liver histology after primary infection and challenge with WHV. (A) Four woodchucks (study group A) were infected (time zero) with 1.9×10^{11} DNase-protected virions of WHV/tm4 inoculum, challenged with the same WHV dose at week 65, and then rechallenged with either 1.9×10^{11} (animals 1/F and 2/F) or 1.9×10^2 (animals 3/F and 4/M) virions at week 80. (B) Four woodchucks (study group B) were infected (time zero) with 1.9×10^{11} virions of WHV/tm4 (animals 5/M and 6/M) or with 1.1×10^{10} virions of WHV/tm3 (animals 7/M and 8/M) and followed for 16 weeks after infection. The appearance and the duration of WHsAg, anti-WHs, anti-WHc, and serum WHV DNA are shown by horizontal bars. The estimated levels of serum WHV DNA are depicted as black bars ($\geq 10^3$ vge/ml), hatched bars ($\leq 10^3$ vge/ml), or white bars (not detectable). The PBMC collected at the time points marked by the short vertical lines at the bottom of panels A and B were analyzed for WHV-specific and generalized T-cell proliferative responses. Samples marked with an asterisk were obtained from all woodchucks except 1/F. The WHV DNA load (A and B) and WHV RNA load (B) in liver biopsies collected at the time points indicated by solid arrow heads are presented as the estimated WHV DNA vge/ μ g of total liver DNA and WHV RNA copies/ μ g of total liver RNA, respectively. Liver morphological alterations are presented as the histological degree of hepatitis graded on a scale of 0 to 3.

FIG. 1—Continued.

the WHc_{97–110} peptide at the same concentrations as those in the adenine incorporation assay or left unstimulated as controls. The plates were incubated for 5 days. The cells were harvested and analyzed in a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The halved CFSE fluorescence in dividing PBMC was determined by using CellQuest Pro software (Becton Dickinson). The cell division index (CDI) was defined by dividing the percentage of cells showing halved CFSE fluorescence after stimulation with WHV antigens by that of cells with halved CFSE fluorescence cultured in the absence of stimulation, as we described before (23). The highest CDI value found at a given time point for any concentration of WHV protein/peptide tested was taken as a measure of WHV-specific T-cell response. The cutoff CDI values of ≥ 3.1 were considered to represent a positive WHV-specific response (23).

CFSE–annexin V-PE–7-AAD assay for simultaneous detection of T-cell proliferation and apoptosis. An assay simultaneously measuring the proliferation of lymphocytes and their apoptotic death was adopted, using ConA and rWhe as stimulators and three-color CFSE–annexin V-phycoerythrin (PE)–7-actinomycin-D (7-AAD) labeling. Briefly, freshly isolated PBMC were labeled with 1 μ M of CFSE, cultured at a density of 5×10^5 cells per well in a 48-well tissue culture plate, and stimulated with either ConA (2.5 and 5 μ g/ml) or rWhe (1 and 2 μ g/ml) as indicated above. The cells were harvested, washed with annexin-labeling buffer (5 mM NaCl, 5 mM KCl, and 2 mM CaCl₂ in 10 mM HEPES), stained for 30 min on ice with 50 μ g/ml of 7-AAD (Molecular Probes) and 50 μ l/ml of R-PE-conjugated recombinant human annexin V (annexin V-PE; Caltag Laboratories, Burlingame, CA), and analyzed by flow cytometry. In addition, to

TABLE 1. Sequences of WHV- and woodchuck gene-specific PCR primers used in this study

Gene	GenBank accession no.	Primer sequence	Amplicon size (bp)
WHV mRNA	AY334075	Forward 5'-ATGCACCCATTCTCTCGAC Reverse 5'-CTGAGCAGCTTGTTAGAGT	221
IFN- α	AF425778	Forward 5'-CTCCATGAGATGACCC Reverse 5'-GGTAGACAGTGATCCT	204
IFN- γ	AF232728	Forward 5'-AGGAGCATGGACACCATCA Reverse 5'-CCGACCCCGAATCGAAG	215
TNF- α	AF333967	Forward 5'-TGAGCACTGAAAGTATGATCC Reverse 5'-TGCTACAACATGGGCTACAG	283
IL-2	DQ272238	Forward 5'-AAGGCTTCTGACCTGG Reverse 5'-CGGGGCGTACTCACAA	185
IL-4	AF333965	Forward 5'-TTTGCTGTCCCAAGAAG Reverse 5'-CCTGGATTCACTCACGG	200
IL-10	AF012909	Forward 5'-AGGCCGTACAGCAAGTGAAG Reverse 5'-ACAACCCTGAGCCAGATTIT	200
IL-12	X97019	Forward 5'-TTGGATTGGCACCCTGACAC Reverse 5'-GCATCTGGCTCAGAACTTCAC	198
CD3	AF232727	Forward 5'-CTGGGACTCTGCCTCTTATC Reverse 5'-GCTGGCCTTTCGGATGGGCTC	536
CD4	EF621765	Forward 5'-GGAGAATAAGAAGATAGAGG Reverse 5'-TCAAGAGTCACAGTCAGG	560
CD14	EF621769	Forward 5'-ACTGTCTGACAATCCT Reverse 5'-CGAGCATCGAGTTGTG	187
CD56	EF621771	Forward 5'-CATCTACAACGCCAATATCG Reverse 5'-GGGAGCTGACCACATC	187
β -Actin	AF232730	Forward 5'-CAACCGTGAGAAGATGACC Reverse 5'-ATCTCTGCTCGAAGTCC	338

monitor the death of lymphoid cells potentially occurring in vivo, freshly isolated PBMC were directly labeled with annexin V-7-AAD without stimulation with ConA or rWHe and analyzed. The authenticity of specific binding of annexin V and 7-AAD was verified by visualizing the intracellular fluorescence distribution (see Fig. 6A) using an Olympus BX 50WI confocal microscopy system (Olympus America, Inc., Center Valley, PA).

The CDI was defined by dividing the percentage of CFSE^{dim} cells after stimulation with rWHe or ConA by the percentage of CFSE^{dim} cells without any stimulation (medium only), as indicated above. A CDI of ≥ 3.1 after rWHe or ConA stimulation was accepted as a positive T-cell response. The level of cell apoptosis was assessed by using CellQuestPro software (Becton Dickinson) and by separating test cells into four quadrants as follows: live cells, i.e., annexin V-7-AAD negative (lower-left quadrant); early apoptotic, i.e., annexin V positive only (lower-right quadrant); late apoptotic, i.e., annexin V and 7-AAD positive (upper-right quadrant); and necrotic, i.e., 7-AAD positive only (upper-left quadrant), as illustrated in Fig. 6A. The total percentage of apoptotic cells was determined by adding the percentages of early apoptotic, late apoptotic, and necrotic cells.

Real-time RT-PCR. PBMC and liver biopsy samples collected prior to and during WHV infection were analyzed for the expression of selected cytokines and cell marker genes by real-time reverse transcription-PCR (RT-PCR) by using a LightCycler (Roche Diagnostics, Mannheim, Germany). For this purpose, 5×10^5 to 1×10^6 PBMC were resuspended immediately after isolation in 1 ml of Trizol reagent (Invitrogen, Auckland, New Zealand) and stored at -20°C . After all experimental samples were collected, RNA was extracted, and 2- μg samples of RNA were reverse transcribed to cDNA, as reported previously (9). The levels of expression of woodchuck IFN- α , IFN- γ , TNF- α , interleukin-2 (IL-2), IL-4, IL-10, IL-12, CD3, CD4, CD14, and CD56 were quantified by using the equivalent of 50 ng of total RNA and the gene-specific primer pairs shown in Table 1, and their levels of transcription normalized against that of woodchuck β -actin (C. S. Guy, P. M. Mulrooney-Cousins, N. D. Churchill, and T. I. Michalak, unpublished data).

To quantify WHV mRNA, cDNA derived from the equivalent of 50 ng RNA, along with serial 10-fold dilutions of complete recombinant WHV DNA as standards (42), was amplified by real-time PCR using WHVc gene-specific primers (Table 1). When WHV RNA was undetectable, 2 μg of cDNA was analyzed by nested PCR-NAH using WHV-specific primers and conditions described before (41).

Statistical analysis. The two-tailed, unpaired Student *t* test, with a 95% confidence interval, was applied to compare the means of the results for the sample groups investigated, and *P* values of <0.05 were considered to be statistically significant.

RESULTS

Serological and WHV DNA profiles after primary and multiple exposures to WHV. Among animals belonging to study group A, three woodchucks developed classical self-limited AH with WHsAg detectable in their sera between 3 and 7 w.p.p.i. (Fig. 1A). Woodchucks 1/F, 2/F, and 3/F seroconverted to anti-WHs between 22 and 37 w.p.p.i., while woodchuck 4/M became transiently anti-WHs reactive 1 week after challenge with WHV, i.e., at 66 w.p.p.i. (Fig. 1A). Anti-WHc appeared at 6 w.p.p.i. and remained detectable during the entire follow-up period in all animals. The level of anti-WHc did not fluctuate noticeably after challenge or rechallenge with WHV. Serum WHV DNA became detectable from 1 w.p.p.i. and persisted until the end of follow-up in all four woodchucks. After clearance of serum WHsAg, the level of circulating WHV DNA declined and usually did not exceed 10^2 vge/ml thereafter, as has been previously reported for animals with secondary occult HBV infection continuing after resolution of AH (10, 41, 42). Serum WHV DNA remained at a similar low level after the second and third injections with WHV (Fig. 1A). Hepatic WHV loads were at 2×10^3 to 8×10^3 vge/ μg of total liver DNA during AH, and then subsided to about 3×10^2 vge/ μg and persisted at an approximately similar level until the end of follow-up (Fig. 1A), as previously observed (10, 41, 42).

In study group B, three animals infected with WHV/tm3 or WHV/tm4 developed immunovirological and WHV DNA pro-

FIG. 2. Kinetics of the WHV-specific T-cell proliferative response against WHV antigens in woodchucks from study group A which were infected, challenged, and rechallenged with WHV. The animals were injected with WHV at the time points indicated by solid arrow heads. Freshly isolated PBMC were stimulated *in vitro* with rWHc, rWHe, rWHx, WHC₉₇₋₁₁₀ peptide, or WHsAg, and their proliferation measured by [³H]adenine incorporation assay. The results are presented as the S.I. *P* values were calculated for peak T-cell reactivity against each WHV antigen in each animal observed during primary (A) and secondary (B) virus exposure in the time periods marked by vertical short lines. The insets show T-cell proliferative responses against the same WHV antigens after rechallenge with WHV as measured by CFSE flow cytometry assay, with the results presented as the C.D.I. The cutoff values for positive responses against rWHc, rWHe, and rWHx were ≥ 3.1 and against WHC₉₇₋₁₁₀ peptide and WHsAg were ≥ 2.1 , as indicated by horizontal dotted lines.

files indicative of a self-limiting episode of AH which were closely comparable to those seen in animals in group A (Fig. 1B), while woodchuck 8/M developed histologically evident mild, protracted hepatitis. In this animal, serum WHsAg appeared at 3 w.p.i. and persisted until the end of follow-up, i.e., 16 w.p.i. Anti-WHs became detectable in animals 6/M and 7/M after clear-

ance of WHsAg from serum around 13 and 10 w.p.i., respectively. Liver biopsies collected at 7 w.p.i. contained WHV DNA loads ranging from 2.4×10^6 to 2.5×10^7 vge/ μ g of total DNA (mean $\pm$ standard deviation, $1.1 \times 10^7 \pm 4.9 \times 10^6$ vge/ μ g) and WHV mRNA at 3.1×10^5 to 5.5×10^6 copies/ μ g of total RNA (mean $\pm$ standard deviation, $2.7 \times 10^6 \pm 1.1 \times 10^6$ copies/ μ g).

Both primary infection and challenge with WHV induce delayed virus-specific T-cell response. In samples from animals from group A, the WHV-specific T-cell proliferation against WHV antigens or the WHC₉₇₋₁₁₀ peptide measured by the [³H]adenine incorporation assay was multispecific and of relatively high magnitude between 10 and 14 w.p.p.i. (Fig. 2). Persistently detectable virus-specific proliferative responses to rWHe, rWHx, and the WHC₉₇₋₁₁₀ peptide first appeared around 6 to 8 w.p.p.i. and preceded those against rWHc and WHsAg, which became detectable at 10 to 14 w.p.p.i. The strong WHV-specific response was evident for up to 30 w.p.p.i. and then subsided and became intermittently detectable with the [³H]adenine incorporation assay until challenge at 65 w.p.p.i. (Fig. 2). In two control animals, the pattern of WHV-specific responses during the acute phase of infection was similar to that described above, and the reactivity remained detectable until termination of the experiment at 112 w.p.p.i. (data not shown). Overall, the virus-specific T-cell response was strong and multispecific during the acute phase of primary infection, with the greatest magnitudes after ex vivo stimulation with rWHe, followed by rWHc or rWHx, and the lowest after stimulation with WHC₉₇₋₁₁₀ or WHsAg.

Challenge of the convalescent woodchucks with the same 1.9×10^{11} vge dose of WHV/tm4 did not induce the expected immediate, virus-specific memory T-cell response (Fig. 2). However, a strong T-cell response to WHV antigens was found in all animals between 5 and 10 weeks after challenge, i.e., 70 to 75 w.p.p.i. The magnitudes of the secondary responses to rWHe ($P = 0.01$), rWHc ($P = 0.03$), and rWHx ($P = 0.001$) were significantly greater than those during the primary infection (Fig. 2). In contrast, the levels of secondary T-cell response against the WHC₉₇₋₁₁₀ peptide ($P = 0.6$) and WHsAg ($P = 0.9$) were comparable to those detected in the primary infection. After rechallenge with either a high (animals 1/F and 2/F) or low (animals 3/F and 4/M) dose of WHV/tm4, samples from all animals demonstrated minuscule or undetectable virus-specific T-cell reactivity when tested with the [³H]adenine incorporation assay (Fig. 2). However, a transient increase in the magnitude of WHV-specific T-cell proliferation was evident when the more-sensitive CFSE flow cytometric assay was applied (Fig. 2, insets).

The generalized proliferative capacity of lymphocytes increases immediately after primary and subsequent exposures to WHV. Almost immediately after the primary injection with WHV, i.e., 1 to 3 w.p.p.i., the lymphocytes acquired from animals in study group A showed a strongly heightened capacity to proliferate in response to ConA, PWM, and PHA (Fig. 3). Then, the response subsided between 6 and 30 w.p.p.i., at the time when the WHV-specific T-cell response appeared, and endured at high levels (Fig. 4). The exception was animal 4/M, with a serum WHsAg-negative but anti-WHC- and WHV DNA-positive infection in which the nonspecific lymphocyte reactivity after the initial lowering increased between 10 and 14 w.p.p.i. During the phase of lowered responsiveness to mitogenic stimuli, temporal increases in this T-cell response occurred. Interestingly, these periodic increases appeared to be synchronized in all animals, as recapitulated in Fig. 4. It also is of note that ConA, PHA, and PWM induced similar profiles of the proliferative response (Fig. 3), suggesting that no particu-

FIG. 3. Kinetics of mitogen-induced (generalized) T-lymphocyte proliferation in woodchucks after primary infection, challenge, and rechallenge with WHV. The same animals (study group A) whose results are shown in Fig. 2 (see the key in Fig. 2) were injected with WHV at the time points indicated by solid arrow heads. The T-cell proliferation in response to five different concentrations of ConA, PWM, or PHA was measured by [³H]adenine incorporation assay. The MMSI values for each mitogen in each animal at the indicated time points were calculated as explained in Materials and Methods. P values were determined as described for Fig. 2.

lar lymphocyte subset was predominantly involved in the periodic increases.

After challenge with WHV at 65 w.p.p.i., this nonspecific T-cell proliferative reactivity was again augmented in all ani-

FIG. 4. Discordance between the kinetics of WHV-specific and generalized T-cell proliferative responses during primary WHV infection and after challenge and rechallenge with WHV. The profiles are compiled from the data generated using a [^3H]adenine incorporation assay to measure T-cell proliferation in response to rWHc, rWHe, rWHx, WHc₉₇₋₁₁₀ peptide, and WHsAg (SI values) and in response to five concentrations of ConA (MMSI values). The mean of the highest SI given by any WHV antigen or that of MMSI in response to any ConA concentration from each of four animals constituting study group A was used to construct the profiles of the WHV-specific and generalized T-cell responses, respectively. Solid and open downward arrows depict peaks of mitogen-induced or WHV-specific T-cell responsiveness, respectively, after primary infection and challenge with WHV. Solid upward arrowheads mark injections with WHV.

mals, with the peak occurring between 1 and 3 weeks following challenge, i.e., 66 and 68 w.p.i. (Fig. 3 and 4). However, the magnitudes of the cell proliferation responses to ConA ($P = 0.2$), PWM ($P = 0.5$), and PHA ($P = 0.1$) were not significantly different from those detected after the first injection with WHV (Fig. 3). This heightened response to mitogens subsided between 68 and 75 w.p.i., which coincided again with the rise of the secondary WHV-specific T-cell response (Fig. 3 and 4). Subsequently, when the virus-specific T-cell reactivity progressively decreased, the T-cell proliferative capacity in response to mitogens rebounded and remained at a high level until 80 w.p.i. when the animals were rechallenged with WHV (Fig. 4). The third injection with either a high or low WHV dose induced another, although lower in magnitude, immediate increase in the T-cell generalized proliferative response (Fig. 3 and 4).

Immediate augmented lymphocyte generalized proliferative response and delayed virus-specific T-cell response are consistently induced by WHV infection. To confirm findings made in the first study by assessing the WHV-specific response using a CFSE flow cytometric assay, as well as to test if another WHV inoculum will induce the same effects in T-cell responses and to systematically evaluate lymphoid cell predisposition to apoptotic death after exposure to WHV, weekly PBMC samples collected from animals constituting study group B were investigated. Thus, the results of the CFSE-based lymphocyte proliferation assay with rWHc confirmed that the primary exposure to WHV induced a delayed virus-specific T-cell re-

sponse which appeared from 7 w.p.i. and persisted until the end of the 16-week follow-up (Fig. 5A). Further, the capacity of the lymphoid cells to proliferate in response to stimulation with a mitogenic stimulus (ConA) was evident again immediately after exposure to WHV, i.e., as early as 1 w.p.i. This response rapidly subsided between 4 and 6 w.p.i., then transiently increased between 7 and 8 w.p.i., and receded to approximately preinfection levels thereafter (Fig. 5B). All four animals belonging to study group B displayed closely comparable patterns of both WHV-specific and ConA-induced T-cell proliferative responses, including animal 8/M, which developed a protracted infection suggesting progression to chronic hepatitis.

Considering the distinct and, for the most part, opposing kinetics of the generalized, mitogen-induced, and WHV-specific T-cell proliferative responses, the progression of acute WHV infection was divided into phases (Fig. 5A and B). Thus, phase A corresponded to the time period prior to WHV infection; phase B, or the preacute phase, occurred between 1 and 3 w.p.i. and was characterized by heightened nonspecific and essentially absent WHV-specific T-cell responses; phase C, or the early acute phase between 4 and 6 w.p.i., was accompanied by subsiding nonspecific and absent virus-specific T-cell responses, and phase D, or the acute phase (7 w.p.i. onwards), was associated with the initially slightly increasing but then normalized mitogen-induced proliferative capacity and with significantly augmented WHV-specific T-cell responses. As shown by the results in Fig. 5C, the magnitude of WHV-

FIG. 5. Patterns of WHV-specific and ConA-induced T-cell responses in group B woodchucks during self-limited acute WHV infection. (A) WHV-specific T-cell response to rWHe was measured by CFSE-based flow cytometric assay. The data are presented as the means $\pm$ standard errors of the means (SEM) of CDIs obtained for PBMC from all four animals that were measured after stimulation with 1 and 2 μ g/ml rWHe at each time point indicated. (B) ConA-induced T-cell proliferative response was measured in the same samples by adenine incorporation assay using twofold dilutions of ConA ranging from 1.25 to 20 μ g/ml. The MMSI was calculated by averaging the SIs from all five ConA concentrations for all four animals at each time point showed. The CDI or MMSI values detected after infection with WHV were compared with those from the same animal before infection (phase A) and are expressed as the *n*-fold increase or decrease. Each data point represents the mean $\pm$ SEM of *n*-fold increase/decrease from all four animals. The preinfection period (phase A) and stages of WHV infection (phases B to D) as defined in Materials and Methods are marked. The cumulative data on WHV-specific (C) and ConA-induced (D) T-cell proliferative responses detected during indicated phases of WHV infection are presented as means $\pm$ SEMs for *n*-fold increases or decreases. The data presented as bars B, C, and D were compared either with those presented as bar A or with each other. Differences marked with one asterisk were significant at a *P* value of <0.05 , with two at a *P* value of <0.005 , and with three at a *P* value of <0.0001 . n.s., nonsignificant.

specific T-cell reactivity during acute infection (phase D) was significantly ($P < 0.0001$) greater than those detected during the preacute (phase B) and early acute (phase C) periods of WHV infection. In contrast, PBMC displayed significantly ($P < 0.0001$) increased magnitudes of mitogen-induced proliferation immediately after exposure to WHV (Fig. 5D), i.e., during the preacute period (phase B). This reactivity declined during the early acute period (phase C), displaying a mean response lower ($P = 0.02$) than that observed prior to infection (phase A), and subsequently rebounded in the acute phase (phase D) to a mean level greater ($P = 0.03$) than that prior to

infection (phase A), but still significantly ($P < 0.005$) lower than that characterizing phase B.

Decline in the generalized proliferation capacity of T cells coincides with increased activation-induced cell death. To recognize a possible reason behind the observed rapid decline in the T-cell proliferative response to mitogenic stimuli seen during the early acute period (phase C) of WHV infection, the rate of apoptotic death of lymphomononuclear cells in weekly PBMC samples from group B animals was evaluated by using the assay employing staining with annexin V-PE, 7-AAD, and CFSE. In preliminary experiments, the authenticity of staining

FIG. 6. Activation-induced apoptosis of lymphoid cells during progression of acute WHV infection. (A) A representative flow cytometry dot plot and a confocal microscopy micrograph showing annexin V-PE- and 7-AAD-labeling patterns in circulating woodchuck lymphoid cells. Staining patterns of annexin V-PE, 7-AAD, and CFSE are visualized with red, blue, and green colors, respectively. (B) Freshly isolated or ConA- or rWHe-stimulated PBMC samples collected prior to inoculation with WHV (phase A) from group B animals or weekly during progression of acute infection were analyzed for the rate of apoptosis by using flow cytometry with annexin V and 7-AAD. The percentages of apoptotic cells were determined in each weekly sample from each of four animals, averaged to find the mean percentage of apoptosis for each phase of infection (phases B to D), and compared with that calculated for PBMC collected prior to WHV infection (phase A), which was taken as 100%. The bars represent the means $\pm$ standard errors of the means of percent increases or decreases in apoptosis for freshly isolated PBMC or those stimulated with ConA at 2.5 and 5 μ g/ml or with rWHe at 1 and 2 μ g/ml. Differences marked with one asterisk were significant at a *P* value of <0.05 , with two at a *P* value of <0.005 , and with three at a *P* value of <0.0001 . n.s., nonsignificant.

of woodchuck lymphoid cells with recombinant human annexin V-PE and 7-AAD was verified by visualizing appropriate sub-cellular compartments with confocal microscopy. As illustrated in Fig. 6A, annexin V, 7-AAD, and CFSE produced cell surface, nuclear, and cytoplasmic staining, respectively, in woodchuck lymphocytes undergoing apoptosis.

To assess whether naturally circulating lymphocytes from different phases of acute WHV infection (Fig. 5) might be differentially predisposed to apoptosis, freshly isolated, unmanipulated, weekly PBMC samples were stained with annexin V

and 7-AAD. The results showed that the fresh cells from different phases did not display any noticeable variations in mean percentage of apoptotic cells in comparison to the rate of apoptosis of the cells collected prior to infection (Fig. 6B). However, when the rate of apoptosis was measured in the same cell samples after stimulation with ConA or rWHe, significantly greater rates and infection phase-dependent changes in apoptotic death were identified (Fig. 6B). Overall, the results indicated that lymphocytes derived from the early acute period (phase C) of WHV infection, when nonspecific T-cell respon-

FIG. 7. Expression profiles of genes encoding cytokines and immune-cell surface markers in serial, unmanipulated PBMC samples collected from the woodchucks investigated. (A) Expression of IFN- α , IFN- γ , TNF- α , IL-4, IL-2, and CD3 in lymphomononuclear cell samples collected from group A animals after challenge and rechallenge with WHV. The solid arrow heads mark the time points of challenge or rechallenge with WHV/tm4. The peak levels of expression of IFN- α (66 to 69 w.p.i.) and IFN- γ and TNF- α (68 to 69 w.p.i.) were compared with those during enhanced WHV-specific response (70 to 74 w.p.i.), and *P* values were calculated as indicated in the legend to Fig. 2. (B) Transcription levels of IFN- α , TNF- α , IFN- γ , IL-12, IL-2, IL-10, IL-4, CD3, CD4, and CD56 in circulating lymphoid cells in different phases of acute WHV infection characterized by distinctive rWHe-specific and Con-A-induced T-cell responses in group B animals. The fold increase/decrease for each gene tested in a given phase of infection (indicated) was calculated and statistically compared as outlined in Materials and Methods. Differences marked with one asterisk were significant at a *P* value of <0.05, with two at a *P* value of <0.005, and with three at a *P* value of <0.0001. n.s., nonsignificant.

siveness profoundly declined but the WHV-specific response had not yet risen, were significantly more prone to activation-induced death than the cells collected soon after infection (phase B) or during AH (phase D).

Cytokine and cell marker gene expression profiles in unmanipulated circulating lymphoid cells. Since the expression patterns of antiviral and proinflammatory cytokines in peripheral lymphoid cells after primary hepadnaviral infection have been

FIG. 7—Continued.

described previously (20, 60), we initially (study group A) examined the expression kinetics after reexposure to WHV. We found that following challenge of animals convalescent from AH with WHV, the heightened expression of IFN- α became immediately evident in sequential PBMC samples collected after challenge, i.e., between 66 and 69 w.p.i. This cytokine transcription then subsided and increased again, but to a lower level, around the time of rechallenge with the same virus inoculum (Fig. 7A). This significantly increased ($P < 0.0001$) expression of IFN- α coincided with the phase of augmented proliferative responsiveness of T-cells to mitogen stimuli (Fig. 7A and Fig. 3, respectively). The transcription of TNF- α ($P < 0.0001$) and, to some extent, IFN- γ ($P = 0.015$) was transiently enhanced between 68 and 69 w.p.i. The mRNA levels of IFN- α , IFN- γ , TNF- α , and, to a lesser degree, CD3 subsided 5 weeks later, i.e., around 70 w.p.i. In general, the enhanced expression of IFN- α , IFN- γ , and TNF- α coincided with the highly augmented T-cell nonspecific proliferative capacity and clearly preceded the reappearance of the WHV-specific T-cell proliferative response, which occurred between weeks 70 and 75 postinfection (Fig. 7A and 2, respectively). The expression profiles of IL-2 and IL-4 did not correlate with the magnitudes of either specific or nonspecific T-cell responses, except for animal 4/M, which displayed increased expression of both these cytokines at the time of greater expression of IFN- γ and TNF- α . The CD14 and CD56 transcription levels measured in serial PBMC samples showed unremarkable profiles (data not shown). Overall, the data revealed a close association between the augmented expression of IFN- α , TNF- α , and, to a lesser extent, IFN- γ in circulating unmanipulated lymphoid cells and their heightened proliferative capacity in response to mitogenic, but not virus-specific, stimuli after challenge with WHV.

To further recognize events accompanying distinctive phases of WHV-specific and nonspecific T-cell proliferative responses accompanying acute WHV infection, the transcriptional activ-

ities of genes encoding selected cytokines and immune-cell subtype-specific markers were quantified by real-time RT-PCR in unmanipulated PBMC collected weekly from animals belonging to study group B. As shown by the results in Fig. 7B, the significantly augmented expression of IFN- α ($P = 0.0001$), IL-12 ($P = 0.005$), IL-2 ($P = 0.005$), and IL-4 ($P = 0.01$) relative to the levels detected prior to infection (phase A) was evident almost immediately after primary exposure to WHV (phase B). This coincided with the elevated expression of CD3 ($P = 0.04$) and CD56 ($P = 0.01$). This selective upregulation of progrowth lymphoid cell cytokines, along with natural killer (NK)- and T-cell-specific markers, was associated with the significantly heightened lymphocyte proliferative responsiveness to stimulation with ConA, but not with WHV (Fig. 5C and D). However, these elevated levels of expression of the cytokines and cell markers indicated above became drastically reduced during the early acute period (phase C), which coincided with the augmented susceptibility of lymphoid cells to undergo activation-induced apoptosis (Fig. 6B). It is important to note that WHV infection failed to induce the expression of either TNF- α or IFN- γ in lymphoid cells during both preacute (phase B) and early acute (phase C) periods (Fig. 7B). Finally, during acute WHV infection (phase D), the levels of expression of IFN- α , TNF- α , IFN- γ , IL-12, IL-2, IL-10, and IL-4 were synchronously upregulated, along with the significantly greater expression of CD3 ($P < 0.0001$) and CD4 ($P < 0.0001$) which coincided with the appearance of the strong WHV-specific T-cell response (Fig. 5A).

WHV replication peaks in lymphoid cells after their increased susceptibility to activation-induced death subsides. As shown by the results for animals from study group B in presented Fig. 8, the WHV mRNA was detectable in serial PBMC samples beginning from the first week postinfection at levels not exceeding 200 copies/ μ g of total RNA. From 5 w.p.i., i.e., at the time of termination of the increased susceptibility of the

FIG. 8. WHV mRNA loads in serial, unmanipulated lymphoid-cell samples collected during different phases of acute WHV infection from group B animals. The total RNA isolated from weekly PBMC samples collected before (phase A) and after inoculation with WHV was reverse transcribed, and virus-specific transcripts quantified by real-time PCR. Phases of infection are indicated at the top. The data represent the means $\pm$ standard errors of the means of WHV RNA loads detected for all four woodchucks at each time point indicated. The horizontal line represents the detection limit of real-time RT-PCR (~ 200 copies/ μ g of total RNA). When negative by real-time RT-PCR, samples were further analyzed by nested RT-PCR-NAH assay (sensitivity 2.5 to 5 copies/ μ g of total RNA).

cells to activation-induced apoptosis, WHV mRNA levels progressively increased in circulating lymphoid cells, peaked at around 11 w.p.i., and then subsided until the end of follow-up. Overall, the results revealed that WHV replication in peripheral lymphoid cells is established as early as 1 week after exposure to a large, liver-pathogenic dose of WHV and progresses at a low level until acute infection becomes serologically and histologically evident (phase D).

DISCUSSION

In the present study, the capacity of lymphocytes to proliferate in response to hepadnaviral antigens and mitogenic stimuli was measured during the preacute and acute phases of experimental WHV infection and after challenge and rechallenge with the same virus. In addition, the predisposition of lymphoid cells to undergo activation-induced apoptotic death and the levels of expression of the cytokines affiliated with the activation of immune cells were quantified in serial samples of circulating lymphoid cells. The study revealed several previously unidentified features of T-cell response accompanying the self-limiting course of hepadnaviral AH and reexposure of the immune host to pathogenic hepadnavirus. Thus, it was found that: (i) a strong lymphocyte activation, evidenced by the heightened proliferative responsiveness to nonspecific stimuli, appears soon after both primary invasion and challenge and, in both situations, prior to the rise of the virus-specific T-cell response; (ii) this temporal lymphocyte hyperresponsiveness to mitogenic stimuli coexists with the augmented expression of cytokines indicative of *in vivo* activation of immune cells and with the impaired transcription of TNF- α and IFN- γ ; (iii) the decline in the lymphocyte nonspecific proliferative responsiveness coincides with the cells' increased susceptibility to activation-induced apoptosis; (iv) the emergence of the virus-specific secondary T-cell response after reexposure to virus is delayed

similarly to its delayed emergence following primary exposure; and (v) the virus-specific and nonspecific lymphocyte proliferative responses display inverse kinetics during the preacute and acute phases of infection and after challenge with hepadnavirus.

In regard to characteristics of the hepadnavirus-specific T-cell response, the results of our study confirmed that self-limited AH is associated with a strong T-cell proliferation reactivity directed toward multiple structural and nonstructural hepadnaviral proteins. This finding is consistent with the data for self-limiting AH type B in humans and chimpanzees (4, 15, 22) and for woodchucks infected with WHV (34, 36). Another feature compatible with the previous observations was the late appearance of the WHV-specific T-cell response after primary exposure, *i.e.*, from 6 w.p.i. onwards. In this context, virus-specific T-lymphocyte proliferative reactivity became detectable from 8 to 12 w.p.i. in HBV-infected humans (4, 63) and 6 to 12 w.p.i. in WHV-infected woodchucks (34). This characteristic of the adaptive T-cell response differs from that seen in other viral infections, where such a response appears in the first 2 weeks after primary exposure (64). Further, WHV-specific T-cell reactivity remained detectable long after the resolution of acute infection, albeit at much-lower magnitudes than during AH (Fig. 2). This finding closely resembles the detection of HBV-specific proliferative and cytotoxic T-cell responses in individuals with a history of resolved AH type B (52), who, like woodchucks exposed to WHV (10, 24, 35, 38, 42), carry low levels of replicating virus for years after the apparent complete resolution of AH (43, 52, 66). In this regard, it is expected that the trace hepadnavirus replication provides sustained antigenic stimuli, maintaining an active antiviral immune response that keeps the persisting virus under relative control. However, this control may fail, leading to clinically evident reactivation of occult infection (reviewed in references 45 and 51).

One of the most intriguing and novel findings in our study was the length of the time required for the WHV-specific T-cell response to reappear after challenge with WHV. It has been well documented that immunocompetent hosts previously exposed to a pathogen swiftly mount a strong secondary adaptive T-cell response that appears within a week following challenge with the same agent. Classical examples are infections with influenza A virus (13, 16, 59) or lymphocytic choriomeningitis virus (17, 46, 55). In our study, instead, the "memory" response in the convalescent woodchucks reexposed to WHV was detected at 5 weeks postchallenge. Further, although the magnitude of this secondary response to the majority of WHV antigens was greater than that in the primary infection, there was no difference in regard to the order in the relative strength of this response toward individual WHV antigens. Overall, the results revealed that both primary and secondary exposures to WHV induced delayed virus-specific T-cell responses with overall similar kinetics. This finding, to our knowledge, is the first of this kind and seems to be a unique feature of hepadnaviral infection.

The examination of a lymphocyte response to mitogens is frequently used to assess the fitness and competence of lymphocytes to perform their immunological functions. This type of responsiveness was found to be transiently or permanently impaired in many microbial infections, and its analysis fre-

quently provided valuable insights into the capacity of the immune system to control the spread of a pathogen and the progression of infection (18, 48, 50, 61). However, this response was only sporadically and fragmentally investigated over the course of HBV infection. In this regard, it has been noticed that during the early phase of serum HBsAg-positive or serum HBsAg-negative HBV infection, lymphocytes displayed a proliferative capacity after stimulation with PHA that was increased relative to that observed during the late phase or in healthy controls (57). The reported data also suggested that acute, as well as chronic, hepatitis B infections are associated with diminished lymphocyte proliferative responsiveness to stimulation with ConA (14), PHA (57), or PWM (12). Our data showed that the proliferative capacity of lymphocytes in response to mitogenic stimuli varied depending on the phase of acute infection, providing a possible explanation for the previously observed variances. The most-interesting findings were that this capacity was significantly augmented almost immediately after WHV infection and subsided prior to the appearance of the adaptive T-cell response (Fig. 4). Surprisingly, a similar sequence of events occurred after the WHV-immune animals were challenged with the same dose of the same virus, which was done more than a year following the first inoculation.

Our finding of the heightened lymphocyte nonspecific proliferation preceding the virus-specific T-cell response is not unique and appears to be consistent with findings for some other viral infections. For example, an acute infection with simian-human immunodeficiency virus (SHIV) in macaques is associated with very early, strong polyclonal activation of lymphocytes followed by the virus-specific T-cell response (61). In this model, the delay in SHIV-specific T-cell reactivity was attributed to inappropriately high generalized activation of lymphocytes, leading to the depletion of CD4⁺ T cells, probably by activation-induced cell death and direct cell killing by the virus, and diminished virus-specific T-cell immunity which, consequently, allowed the virus to spread in the early phase of infection. In this context, it is of note that WHV is also a lymphotropic virus which invariably infects the immune system, even when the liver is not affected, as observed for woodchucks inoculated with WHV doses equal to or below 10³ virions or for offspring born to woodchuck dams convalescent from AH (9, 41). Also, WHV invasion of the lymphatic system occurs soon after inoculation, as evidenced by the detection of WHV DNA and its replicative intermediate, covalently closed circular DNA, in lymphoid cells as early as 3 days postinoculation with either liver-pathogenic or low, liver-nonpathogenic doses of WHV (C. S. Guy, P. M. Mulrooney-Cousins, and T. I. Michalak, unpublished data).

To gain further insights into the nature of the immune responses accompanying the early phase of hepadnaviral infection, the transcription levels of the cytokines affiliated with the activation of immune cells and those of the markers specifying individual immune-cell subtypes were quantified in serial samples of circulating lymphoid cells. The cytokine expression patterns revealed were analyzed in the context of the profiles of T-cell-nonspecific and WHV-specific responses and the susceptibility of the cells to activation-induced apoptotic death. In this regard, it is of note that viral infections normally induce an immediate type I interferon response, i.e., IFN- α and IFN- β ,

which precedes the production of other cytokines, such as TNF- α , IL-1, IL-12, and IL-2, by antigen-presenting cells (APC) and NK cells (27). Together, these cytokines mediate the induction of the effective innate response and facilitate the prompt development of adaptive immunity. It has also been shown that during the initial phase of infection, activated NK cells can eliminate invading virus both by killing infected cells and via a noncytopathic pathway mediated by IFN- γ (2, 21, 27). At the same time, around 1 w.p.i., activated APC acquire the ability to promote the virus-specific T-cell immune response which inhibits infection from then onwards (2). In regard to hepadnaviral infection, an investigation with chimpanzees infected with a massive dose of HBV ($\sim 10^8$ virions) failed to reveal upregulated expression of the genes affiliated with the activation of the innate immune response for up to 6 to 10 w.p.i. when measured by microarray analysis of liver samples (65). Similarly, the intrahepatic expression of IFN- γ and TNF- α , along with CD3-positive lymphomononuclear cell infiltration, was found to be delayed until the acute phase of WHV infection, as determined by RT-PCR analysis (24, 47). However, these measurements were initiated not earlier than 1 w.p.i. and were performed, in some instances, by using methods of relatively low sensitivity, raising a concern that the early response or that of a lower magnitude was overlooked. In fact, the recently completed analysis of the expression profiles of the genes affiliated with the active innate immune response in serial liver biopsies collected from 1 h post-WHV infection, by applying highly sensitive real-time RT-PCR assays, revealed upregulated transcription of IFN- γ and IL-12 and activation of APC and NK cells as early as 3 to 6 h and activation of NK T cells at 48 to 72 h postinfection with WHV (C. S. Guy et al., unpublished data). Therefore, hepadnavirus induces an intrahepatic innate response but this response arises almost immediately after infection, is transient, and is not succeeded by the prompt appearance of the local adaptive T-cell immunity.

Peripheral lymphoid cells collected weekly from WHV-infected animals in our study demonstrated a transient but significantly augmented expression of IFN- α , along with IL-12, IL-2, IL-4, CD3, and CD56, between 1 and 3 w.p.i. (Fig. 6B). The same cells, unexpectedly, did not display enhanced expression of TNF- α and IFN- γ for up to 6 w.p.i., i.e., when AH had fully developed. The identified prolonged absence of TNF- α transcription in immune cells in the early WHV infection may have important consequences, due to the multiple immunological functions of this cytokine. Among others, TNF- α , together with IFN- α , drives the differentiation of dendritic cells (DC) from precursors (25, 58), while DC in the presence of IFN- α alone acquire an improper mature phenotype characterized by impaired antigen-specific and *allo*-stimulatory capacities (11, 32). Furthermore, the neutralization of TNF- α by specific antibody administered to lymphocytic-choriomeningitis virus-infected mice abolished the virus-induced activation of DC (26), while DC cultured in the presence of both TNF- α and IFN- α acquired a phenotype comparable to that induced *in vitro* by infection with herpes simplex virus (26). Taken together, the prompt expression of TNF- α results in the appropriate maturation of DC, the effective activation of APC, and the expeditious rise of effective adaptive T-cell immunity (58). Nonetheless, along with the activation of DC and NK cells, the initiation of effective antiviral T-cell immunity also requires the

synchronous expression of other cytokines, such as IFN- α , IL-12, IL-2, IL-4, and IL-10 (2, 49). Thus, the absence of TNF- α and the nonsynchronous expression of some of the other cytokines (e.g., IL-10; Fig. 7B) during the early phase of WHV infection could translate into inappropriate differentiation and activation of DC, which in turn may fail to promote the appropriate presentation of WHV antigens and postpone the priming of the antiviral adaptive T-cell response.

On the other hand, an early IFN- α response, along with DC-derived cytokines induced by viral infection, initiates proliferation and IFN- γ production in NK cells (6, 28). In turn, this cytokine enhances the NK cell cytotoxic function, as well as shaping the development of the antigen-specific Th1 immune response (2, 3, 27, 49). Therefore, the absence of IFN- γ transcription in circulating lymphoid cells for up to 6 weeks after the administration of WHV further supports the notion of aberrant activation of the innate response in the initial phase of hepadnaviral infection. This defect may further hamper the early elimination of hepadnavirus, as well as contribute to postponement of the rise of the virus-specific T-cell response.

We found that lymphoid cells displayed significantly higher susceptibility to activation-induced apoptosis at the time of decreased proliferative responsiveness to mitogenic and WHV-specific stimuli. To determine a possible reason behind this rapid decline in lymphoid cell proliferative responsiveness in the early acute WHV infection, we measured the rates of apoptosis in freshly isolated weekly PBMC samples and in the same samples after *ex vivo* stimulation with ConA or rWHe. The results showed that the rates of apoptosis in unmanipulated lymphoid cells were essentially the same during different phases of the development of acute WHV infection. A comparable observation was reported for unmanipulated, circulating lymphocytes during infection with human immunodeficiency virus (HIV) (19). In contrast, the same woodchuck cells exposed to ConA or rWHe displayed meaningfully different susceptibility to apoptosis depending upon the phase of acute WHV infection, defined by the contrasting kinetics of nonspecific and WHV-specific T-cell responses (Fig. 5 and 6B). Taken together, the results of the analysis showed that the decline in the cells' proliferative responsiveness to mitogenic stimuli in the early WHV infection coincided with the cells' significantly greater susceptibility to activation-induced apoptotic death. Thus, this cooccurrence resembled the mechanism proposed for the depletion of nonspecifically activated lymphocytes during acute SHIV infection (61).

The increased susceptibility of cells to activation-induced cell death found in our study was accompanied by reduced IFN- α , IL-2, and IL-12 transcription and by upregulated expression of IL-10 in circulating lymphoid cells. In this regard, seemingly comparable activation-induced death of lymphoid cells was reported during experimental infection with SHIV (61) and during HIV infection (19) and was attributed to abnormal expression of cytokines or to defective antigen presentation by APC (8). It was also observed that CD4⁺ and CD8⁺ T cells derived from HIV infection and treated *ex vivo* with PWM or anti-CD3 antibodies in the presence of IL-12 demonstrated enhanced survival, while those treated in the same way in the presence of IL-10 displayed an increased susceptibility to apoptosis (8). Further, during the acute phase of infection with Epstein-Barr virus, an increased susceptibility

of activated CD45RO⁺ T cells to apoptosis in culture was shown to be reversed by the addition of exogenous IL-2 (1). These findings resemble those encountered in early WHV infection (phase C), where the decreased expression of cytokines, especially IL-12 and IL-2, could initiate defective T-cell activation with higher susceptibility of the lymphocytes to activation-induced death (Fig. 6B and 7B). This may represent a mechanism by which improperly activated lymphocytes are eliminated *in vivo*. On the other hand, a contribution of WHV to the cells' enhanced susceptibility to apoptotic death was unlikely, since the peak of virus replication occurred in lymphoid cells when the acute infection became well established (phase D; Fig. 8).

Based on the data acquired in the current study, we postulate that the early aberrant lymphocyte activation, followed by apoptotic death of the activated cells and coinciding with a defective expression of the cytokines critical for the prompt initiation of the virus-specific T-cell response, is responsible for the postponement of adaptive T-cell responses in hepadnaviral infection. The identification of a mechanism by which hepadnavirus induces the immediate, defective lymphocyte activation will require further investigation. Similarly, a contribution of the delayed virus-specific T-cell response to the intrinsic ability of hepadnavirus to escape from sterilizing elimination by the immune system, leading to the establishment of either symptomatic or occult persistent infection, needs to be explained. Overall, the results of the present study throw a new light on the features of immune-cell responsiveness accompanying hepadnaviral infection. Because of the high compatibility between the virological and pathogenic properties of WHV and HBV and strong parallels in the induced antiviral responses, the current data should contribute to our better understanding of the immunological events underlying a prolonged incubation period and the delayed initiation of AH in HBV infection and, possibly, to the design of methods of halting the development of chronic hepatitis.

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