Infection with hepatitis B virus (HBV) often results in an acute bout of hepatitis followed by clinical recovery, but progress to chronic infection and disease such as liver cirrhosis and hepatocellular carcinoma is sometimes observed. Several studies indicate that cellular immune responses to HBV proteins have a major influence on the clinical course of HBV infection: vigorous and multispecific T helper (Th)-cell and cytotoxic T-lymphocyte responses to HBV surface protein (HBsAg), core protein (HBcAg), polymerase protein, and X protein (HBxAg) have been associated with recovery from acute HBV infection and viral clearance (4, 11, 12, 17, 18, 21, 26, 29; for reviews, see references 6 and 10). In contrast, low or undetectable T-cell responses to these proteins were associated with viral persistence and chronic hepatitis (3, 27; reviewed in references 6 and 13). However, the exact immunologic mechanisms which contribute to viral elimination or persistence during the natural course of hepadnavirus infection are unknown. Because there are no clinical symptoms immediately after HBV transmission, T-cell-mediated immune responses during the incubation period and the early phase of hepatitis in patients are difficult to analyze. It is also unknown to what extent a long-lasting T-cell response may support protection from viral reinfection after resolution of an acute, self-limited HBV infection (30, 34).

Woodchucks (Marmota monax) infected with woodchuck hepatitis virus (WHV) represent the animal model closest to humans for studying the cell-mediated immune response during hepadnavirus infections. WHV and HBV exhibit a high degree of homology in their nucleotide sequences, genomic organizations, and replication and expression mechanisms (5, 8, 14, 19, 35, 39). Furthermore, the humoral immune responses during the course of acute or chronic infections of HBV-infected humans and WHV-infected woodchucks are similar (31–33, 41).

There are similarities in the cellular immune responses of woodchucks to WHV and the responses of humans infected with HBV. In WHV-infected adult woodchucks, strong T-cell responses to surface protein (WHsAg) and recombinant core protein (rWHcAg) are found (7, 23, 25). The T-cell responses in these woodchucks to WHcAg are predominantly to specific peptides of the antigen, whereas in chronically WHV-infected woodchucks an undetectable or weak T-cell response to these proteins and peptides is observed (23–25). The importance of T-cell responses for the elimination of WHV has been demonstrated by protective immunization with a single WHcAg peptide (amino acids 97 to 110) which contains a major T-cell epitope (23). It was demonstrated in vitro that proliferating cells from these animals were T cells, as shown by staining with a monoclonal antibody against CD3ε (23). Although further characterization of T cells in the woodchuck is not yet possible, the proliferation assays were analogous to those used in humans and the use of linear peptides suggested that the T cells were Th cells.

The experimental WHV infection of woodchucks is an important model with which to examine the role of cellular immune responses during the early phase of acute hepadnavirus infection. During this crucial phase, the course of infection...
resulting in viral elimination or persistence may be determined. This study investigated the kinetics of T-cell responses to WHsAg, rWHcAg, and WHcAg peptides during the early acute phase of self-limited WHV infection. rWHcAg-specific polyclonal T-cell lines were established at different times after infection, and their responses to WHAg-related peptides were measured. Recognition of rWHcAg and several WHcAg-related peptides was shown to occur over a period of 6 months after inoculation, as was recognition of the immunodominant WHAg epitope (amino acids 97 to 110) described previously (23). The T-cell responses after convalescence (1 year after infection) and upon challenge with WHV were also examined. No T-cell proliferation in response to WHV antigens could be detected 1 year after infection. Reactivation of the T-cell responses to viral antigens and to the previously recognized WHAg epitope was demonstrated after challenge with WHV.

MATERIALS AND METHODS

Animals. Four WHV-negative woodchucks, trapped in the state of New York, were purchased from North Eastern Wildlife (Ithaca, N.Y.) and maintained in our facility. Before experimentation, all the animals were clinically examined and tested for parasitic infections including intestinal worms. Serologic testing was also performed, and all adult animals, of either sex, were found to be negative for WHV DNA, WHAg, and antibodies against WHsAg (anti-WHs) and WHAg (anti-WHAg).

Serologic testing, virus detection, and liver function assay. Serologic testing for markers of WHV replication during the course of acute WHV infection and convalescence and after viral challenge was performed weekly. WHsAg, anti-WHs, and anti-WHc were determined by an enzyme-linked immunosorbent assay (ELISA) as described previously (36, 37). WHV DNA was detected by PCR with two WHV core gene-specific oligonucleotide primers (nucleotides 2015 to 2038 and 2570 to 2595) (23) or by nested PCR with four oligonucleotide primers (nucleotides 2015 to 2038, 2630 to 2656, 2129 to 2148, and 2597 to 2618) after extraction of DNA from serum or from PBMC. Additionally, WHV DNA was detected by a dot blot technique as described previously (36). Sorbitol dehydrogenase (SDH) activity, a marker of acute liver damage in humans (2, 43) was detected by a dot blot technique as described previously (36).

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Kinetics of WHV serological markers, WHV DNA, and T-cell responses to viral antigens during acute, self-limited WHV infection. Markers of WHV infection and T-cell proliferation in response to WHV antigens were analyzed in four experimentally WHV-infected adult woodchucks (NW7029, NW7030, NW7031, and NW7032) to characterize the appearance and duration of humoral and cellular immune responses to WHsAg, rWHcAg, and WHAg-related peptides during the incubation period and the early phase of acute WHV infection. Humoral and cellular immune responses were determined weekly for a total of 12 weeks.

Markers of WHV infection. Woodchucks NW7029, NW7030, and NW7032 showed a similar pattern of viral markers, i.e., appearance and duration of viremia (WHV DNA, WHsAg) and onset of convalescence (anti-WHs and anti-WHc). WHV DNA and WHsAg were detected in the serum from week 2 or 3 to week 7 (Fig. 1). Anti-WHc and anti-WHs were detected in the serum beginning at week 4 or weeks 5 to 6, respectively, and continued throughout the study (more than 52 weeks). A positive signal for anti-WHC at week 1 is a result of infection with an inoculum containing a high titer of anti-WHC. The SDH level in serum increased from normal values (86 to 182 IU) beginning at week 4 and reached its peak value at week 7 (1,185 to 1,325 IU). The SDH activity subsequently decreased and returned to normal values by week 9.

The pattern of viremia and increase in SDH levels were different in woodchuck NW7031 from those in the other three animals (Fig. 1). Although the onset of viremia was similar, WHV DNA and WHsAg were detected in the serum of NW7031 for an additional 3 weeks. Anti-WHC was detected in serum beginning at week 4, but anti-WHs was not detected until week 8. Both antibodies were then detected throughout the remainder of the study. SDH levels began to increase at

RESULTS

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weeks 5 to 6 (212 IU) to a peak level at week 8 (1,219 IU) and declined to normal values by week 11.

Cellular immune responses to WHV antigens. (i) T-cell response to WHsAg. By using both a BrdU assay and a [2-3H] adenine incorporation assay, the first T-cell response detected was directed against WHsAg in woodchucks NW7029, NW7030, and NW7032 (Fig. 1). This T-cell response was detected 3 weeks after experimental infection and correlated with the detection of WHV DNA and WHsAg in the serum as well as with an increase in SDH levels. A maximum T-cell response to WHsAg was observed at week 4. The mean SI was 2.3 to 2.6 in the BrdU assay and 4.2 to 4.9 in the [2-3H]adenine assay. By weeks 5 to 6, the T-cell response to WHsAg decreased, and it was below the cutoff value by week 8. The decrease in the T-cell response to WHsAg coincided with the detection of anti-WHs in serum (weeks 5 to 6). Clearance of WHV DNA (week 8) and normalization of SDH levels (week 9) followed shortly. Some differences in the course of the T-cell response to WHsAg were evident between woodchuck NW7031 and the other three animals (Fig. 1). Depending on the assay, a T-cell response to WHsAg was detected 1 to 3 weeks later in the course of infection beginning on weeks 4 to 6. The T-cell response reached its maximum level 7 weeks after infection (mean SI, 2.5 in the BrdU assay and 4.9 in the [2-3H]adenine assay). Similar to the other three animals, the T-cell response decreased afterwards (weeks 8 and 9), coincident with the detection of anti-WHs (week 8). Clearance of WHV DNA and normalization of SDH levels were observed at week 11.

As a control for the observed specific T-cell response to WHsAg that was purified from the sera of chronically WHV-infected woodchucks, the studied animals were tested in parallel for their response to pooled serum from WHV-negative woodchucks. Neither the four woodchucks (NW7029, NW7030, NW7031, and NW7032 [data not shown]) nor several WHV-negative woodchucks (Table 1) showed a T-cell response to these serum in the [2-3H]adenine assay. Stimulation of PBMC from WHV-negative woodchucks with WHsAg led to no proliferative response (Table 1).

### TABLE 1. PBMC response of WHV-negative woodchucks to serum, WHV antigens, and WHcAg peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>PBMC response (mean SI) of WHV-negative woodchuck&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>1</td>
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<tr>
<td>WHV-negative serum</td>
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<tr>
<td>WHsAg</td>
<td>1.3</td>
</tr>
<tr>
<td>rWHcAg</td>
<td>1.6</td>
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<tr>
<td>Peptide 97–110</td>
<td>1.7</td>
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<tr>
<td>Peptide 129–140</td>
<td>1.5</td>
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</tbody>
</table>

<sup>a</sup> Responses to medium supplemented with 10% pooled serum from WHV-negative woodchucks, 2 μg of WHsAg per ml, 1 μg of rWHcAg per ml, 1 μg of peptide 97–110 per ml, and 1 μg of peptide 129–140 per ml were analyzed by [2-3H]adenine incorporation. PBMC (5 × 10⁴) were obtained from WHV-negative woodchucks. The results are presented as mean SI of triplicate determinations. The mean cpm for the controls was 2,615 ± 1,473.
(ii) T-cell response to rWHcAg. The T-cell responses to rWHcAg in woodchucks NW7029, NW7030, and NW7032 were detected 1 week after the T-cell response to WHsAg (Fig. 1). The T-cell response to rWHcAg reached maximal levels at week 6 and decreased thereafter (weeks 7 to 9). The maximum mean SI was 2.9 to 3.3 in the BrdU assay and 6.8 to 7.5 in the [2-3H]adenine assay. As expected, T-cell response was seen at weeks 9 to 10 (mean SI became detectable at weeks 4 to 6 (Fig. 1). The maximum mean SI was 2.9 to 3.3 in the BrdU assay and 6.8 to 7.5 in the [2-3H]adenine incorporation.

(iii) T-cell response to WHcAg-derived peptides. In addition to the T-cell responses to WHsAg and rWHcAg, the T-cell response to the peptide from amino acids 97 to 110 (peptide 97–110) representing an immunodominant epitope of the WHcAg (23), was monitored. As a control for the peptide-specific proliferative response, based on previous experiments (23), the WHcAg peptide 129–140 was used. Stimulation of PBMC from WHV-negative woodchucks with peptide 97–110 or 129–140 resulted in no T-cell response (Table 1). T-cell responses to peptide 97–110 were detected in woodchucks NW7029, NW7030, and NW7032 3 to 4 weeks after infection (Fig. 1). A maximum T-cell response was seen at week 6 and decreased thereafter (weeks 7 to 10). The maximum mean SI was 4.2 to 4.9 in the BrdU assay and 8.2 to 9.1 in the [2-3H]adenine assay.

A T-cell response to peptide 97–110 in woodchuck NW7031 became detectable at weeks 4 to 6 (Fig. 1). The maximum T-cell response was seen at weeks 9 to 10 (mean SI = 4.2 in the BrdU assay and 8.6 in the [2-3H]adenine assay). As expected, stimulation with peptide 129–140 did not result in T-cell proliferation in the tested animals at any time during acute WHV infection (Fig. 1). Based on these results, we decided to assess T-cell responses in the following experiments, e.g., of polyclonal T-cell lines or after WHV reinoculation, only by [2-3H]adenine incorporation.

Recognition of WHcAg epitopes during acute WHV infection and convalescence. Epitopes recognized by Th cells are major histocompatibility complex (MHC) class II restricted (42), and it has previously been shown that T cells of outbred woodchucks recognize different epitopes of WHcAg (23). However, one epitope located between amino acids 97 and 110 appeared promiscuous, since it has so far been recognized by all the woodchucks tested, whereas several peptides from panel A (peptides 1–20, 28–47, 28–57, 70–89, 90–109, 100–119, 112–131 and 120–139) whereas woodchuck NW7031 showed PBMC proliferation only to three peptides of panel A (Table 2). Four peptides of panel B (peptides 97–110, 100–113, 111–124, and 120–131) induced proliferation of PBMC from the four woodchucks tested, whereas several peptides from panel A (peptides 15–34, 50–69, 61–80, 82–101, 131–150, and 146–165) were not stimulatory for PBMC from any of these animals.

Comparing epitopes which were recognized by PBMC and by T cells of rWHcAg-specific polyclonal T-cell lines 6 weeks after experimental infection, we demonstrated that the number of peptides recognized did not differ generally (Fig. 2). Two exceptions were found: the T cells of woodchucks NW7029 and NW7032 recognized only peptide 112–131 and 50–69, respectively.

To test whether the repertoire of epitopes recognized during the incubation period, the acute phase, and convalescence varied, rWHcAg-specific polyclonal T-cell lines were established at weeks 6, 12, 18 (at this time only from woodchucks NW7030 and NW7031), and 24 after experimental WHV infection. These lines were stimulated with peptides of both panel A and B, and it was found that all stimulatory peptides recognized by rWHcAg-specific T cells of woodchucks NW7029 and NW7032 at week 6 also induced T-cell responses during the following weeks, although their stimulatory effects decreased during convalescence. Only two peptides (peptides 38–57 and 120–131) did not induce the stimulation of T-cell lines from these woodchucks at week 24.

T-cell responses at week 24.

### Table 2. PBMC response to WHcAg epitopes during acute WHV infection

<table>
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<tr>
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*PBMC responses 6 weeks after experimental WHV infection to 1 μg of WHcAg peptides per ml were analyzed by [2-3H]adenine incorporation. *PBMC (5 × 10⁴) were obtained from woodchucks NW7029, NW7030, NW7031, and NW7032. Results are presented as mean SI of triplicate determinations. SI values of ≥3.1 are indicated in bold type. The mean cpm for the controls was 2,863 ± 1,397.
FIG. 2. T-cell responses of PBMC 6 weeks after experimental WHV infection and of rWHcAg-specific T-cell lines established at 6, 12, 18, and 24 weeks postinfection to 1 μg of rWHcAg or WHcAg peptides per ml were analyzed by [2-3H]adenine incorporation. PBMC (5 × 10⁴) were obtained from woodchucks NW7029, NW7030, NW7031, and NW7032 6 weeks after infection. T cells (5 × 10⁴) were derived from polyclonal T-cell lines established at weeks 6, 12, 18, and 24 by continuous stimulation with rWHcAg. Results are presented as mean SI of triplicate determinations. The mean cpm for the controls was 7,043 ± 3,368.
BSA induced no stimulatory effects on these rWHcAg-specific T-cell lines (data not shown).

T-cell response to WHV antigens 1 year after infection. During the acute phase of WHV infection (week 6), PBMC of all woodchucks recognized WHsAg, rWHcAg, and certain WHcAg epitopes. To determine whether these woodchucks possess a long-lasting cellular immune response, PBMC from woodchucks NW7030 and NW7031 were stimulated with viral proteins and WHcAg peptides 1 year after the experimental WHV infection. At this time, both animals were positive for anti-WHs and anti-WHc and negative for WHsAg. WHV DNA was detectable neither in the sera nor in the PBMC by nested PCR (data not shown). Furthermore, the same WHcAg peptides, which no longer induced T-cell proliferation during the convalescence period (week 24 [Fig. 2]), also remained unstimulatory after challenge with WHV (Fig. 5).

Peptide stimulation of PBMC and T cells of rWHcAg-specific T-cell lines obtained 1 week after viral challenge confirmed the reactivation of the cellular immune response and also showed a loss of recognition of certain WHcAg epitopes (peptides 38–57, 120–131, and 120–139) (Fig. 5). T cells obtained from both animals after viral challenge recognized the same subset of WHcAg epitopes that were recognized during the period of an acute WHV infection (Fig. 2). Furthermore, the same WHcAg peptides, which no longer induced T-cell proliferation during the convalescence period (week 24 [Fig. 2]), also remained unstimulatory after challenge with WHV (Fig. 5).

**DISCUSSION**

Appropriate Th and cytotoxic T-lymphocyte responses during the early phase of an acute HBV infection are regarded as

For peptide 97–110, and for woodchuck NW7031 the SI was 3.2, 3.4, and 4.7, respectively. Control peptide 129–140 did not stimulate T cells from either animal. T-cell responses were detected for 2 weeks and decreased thereafter, approaching cutoff values by week 4. At the time of viral challenge, both woodchucks were positive for anti-WHs and anti-WHc and negative for WHsAg. WHV DNA and WHsAg were not detectable in the sera by nested PCR or ELISA, respectively, at the time of challenge and thereafter.

Peptide stimulation of PBMC and T cells of rWHcAg-specific T-cell lines obtained 1 week after viral challenge confirmed the reactivation of the cellular immune response and also showed a loss of recognition of certain WHcAg epitopes (peptides 38–57, 120–131, and 120–139) (Fig. 5). T cells obtained from both animals after viral challenge recognized the same subset of WHcAg epitopes that were recognized during the period of an acute WHV infection (Fig. 2). Furthermore, the same WHcAg peptides, which no longer induced T-cell proliferation during the convalescence period (week 24 [Fig. 2]), also remained unstimulatory after challenge with WHV (Fig. 5).
adenine incorporation (20, 23) due to low \([\text{3H}]\)thymidine up-
assay had higher sensitivity in that the T-cell could be monitored by both assays (Fig. 1). However, the woodchuck T-cells to WHsAg, rWHcAg, and WHcAg peptides in parallel to compare their sensitivity (25). The response of kinetics of T-cell responses after infection, we used both assays assays is incorporated into cellular DNA has not yet been examined. T-cell responses were assessed by BrdU and \([2-\text{3H}]\)adenine after experimental WHV infection of adult woodchucks were determine their role in disease progression.

\[\text{FIG. 5. T-cell responses of PBMC (5 \times 10^4) and rWHcAg-specific T-cell lines (5 \times 10^4) 1 week after viral challenge to 1 \mu g of rWHcAg or WHcAg peptides per ml were analyzed by [2-\text{3H}]adenine incorporation. PBMC were obtained from woodchucks NW7030 and NW7031 1 week postchallenge. T cells were derived from polyclonal T-cell lines established 1 week after viral challenge by continuous stimulation with rWHcAg. Results are presented as mean SI of triplicate determinations. The mean cpm for the controls was 4,691 \pm 2,352.}\]

were detected 3 weeks after WHV inoculation (Fig. 1). The maximum T-cell response to WHsAg occurred when WHsAg was detected in serum and decreased upon seroconversion to anti-WHs. The development of anti-WHs, which is regarded as a virus-neutralizing antibody (40), was associated with the elimination of WHsAg from serum. These findings suggest that T-cell responses to WHsAg occur prior to liver cell damage, as shown by the presence of normal SDH levels in serum (Fig. 1). WHsAg may be secreted at high abundance early after infection and therefore is the first antigen presented to T cells by MHC class II molecules of APC. In addition, the differential abundance of WHsAg and WHcAg could explain why T-cell responses to rWHcAg and the corresponding immunodominant epitope (peptide 97–110) became detectable later than the WHsAg-specific T-cell response (Fig. 1).

The T-cell response in woodchucks to recombinant WHV core protein was similar to the response observed in HBV-infected patients, whose HBcAg-specific T-cell responses were stronger and occurred later during the course of infection than did the HBsAg-specific T-cell responses (12, 17, 21). This was likely for the stronger T-cell proliferation to major epitopes of the HBcAg compared to the entire rHBcAg. In all four woodchucks, stimulation of T cells with peptide 97–110 resulted in a greater proliferation than with rWHcAg. The maximum T-cell responses to rWHcAg and peptide 97–110 coincided with the peaks of SDH activities.

The more pronounced T-cell response to rWHcAg than to WHsAg may depend on the higher immunogenicity of this internal viral protein (23, 28). The vigorous T-cell response to WHcAg epitopes (e.g., peptide 97–110) is probably based on their linear features facilitating internalization and presentation by MHC class II molecules of APC.

The T-cell responses to the peptides described previously (23) and used in this study indicate that a variety of epitopes, located throughout the entire core protein, are recognized. The subset of WHcAg epitopes recognized by each animal was different, however (Table 2) (23). One reason for this may be that the animals used in these studies were outbred and unrelated to each other in terms of MHC. This was confirmed by a one-way mixed lymphocyte reaction (data not shown) and characterization of MHC class I patterns (unpublished results). Peptide 97–110 was recognized by all four woodchucks tested in this study, strengthening previous findings that this epitope is promiscuous and immunodominant (23). So far, epitopes covering amino acids 15 to 34, 50 to 69, 61 to 80, 82 to 101, 131 to 150, and 146 to 165 were not recognized (Table 2).

The T-cell response in woodchucks to recombinant WHV core protein was similar to the response observed in HBV-infected patients, whose HBcAg-specific T-cell responses were stronger and occurred later during the course of infection than did the HBcAg-specific T-cell responses (12, 17, 21). This was likely for the stronger T-cell proliferation to major epitopes of the HBcAg compared to the entire rHBcAg. In all four woodchucks, stimulation of T cells with peptide 97–110 resulted in a greater proliferation than with rWHcAg. The maximum T-cell responses to rWHcAg and peptide 97–110 coincided with the peaks of SDH activities.

The T-cell responses to rWHcAg and several epitopes were present beginning 6 weeks after experimental WHV infection.
and continued to be detected throughout convalescence (weeks 12, 18, and 24). Similar to results with rWHcAg, peptide 97–110 and peptides 100–113, 100–119, and 112–131 were recognized by the T cells of all woodchucks throughout this study. Further WHcAg epitopes, e.g., peptides 1–20, 70–89, and 90–109, which were recognized by woodchucks NW7029, NW7030, or NW7032 at week 6 were also stimulatory at week 24. T-cell proliferation in response to additional WHcAg epitopes during the follow-up was not observed. However, several peptides, e.g., peptides 38–57, 120–131, and 120–139 which induced a strong T-cell response at weeks 6 to 18, were no longer stimulatory for T cells after convalescence (week 24). Consistent responses to different epitopes during a 24-week follow-up have not been detected in studies on human patients (9, 17).

Recent studies have shown a long-term persistence of HBV-specific Th-cell and CTL responses in patients after recovery from HBV infection (30, 34). These results may be due to T-cell memory after initial development of a vigorous T-cell response which led to resolution of HBV infection. They may also be due to the continuous stimulation by residual virus, replicating at very low levels in extrahepatic sites, as demonstrated by the detection of HBV DNA in the sera and/or PBMC of some patients. In contrast to these findings, WHV-specific T-cell responses or WHV DNA were not detected 1 year after the acute self-limited WHV infection in the four woodchucks tested (Fig. 3). This could be due to the short recovery period after infection or to the limited number of animals. However, a decrease in the Th-cell responses to HBV nucleocapsid antigens has been observed in acutely HBV-infected patients simultaneously with or shortly after resolution of infection (11, 12). Especially if HBV DNA was not detected in the serum, the Th-cell and CTL responses to HBV proteins were low (30, 34). These results are explained by the down-regulation of the frequency of effector cells toward the end of a successful immune response (1, 38).

The woodchuck model presents a unique opportunity to investigate the cellular immune response upon reexposure to hepadnavirus infection. To test for the presence of memory T cells, which may become reactivated upon challenge with the virus, two animals were inoculated with 10⁶ woodchuck ID₅₀ 1 year after recovery from the initial infection. T-cell proliferation in response to WHV antigens was demonstrated 1 week after challenge in both animals (Fig. 4). This T-cell response was detected for 2 to 3 weeks and decreased thereafter. Although circulating anti-WHs may have neutralized most virus particles, a small number of hepatocytes could have been infected, resulting in a limited and low-level viral replication that was responsible for the reactivation of T cells. Interestingly, the reactivated T-cell responses were directed against the same WHcAg epitopes observed during convalescence after the primary WHV infection (Fig. 2 and 5). The similar pattern of T-cell responses found in these woodchucks suggests a hierarchy of specificity in epitope recognition that is maintained unchanged after convalescence until reexposure to the virus. In conclusion, these results demonstrate the development of T-cell responses in the woodchuck directed against WHcAg, rWHcAg, and distinct epitopes of the WHcAg during the early phase of infection and show that they are closely associated with viral clearance and are retained after convalescence, contributing to long-lasting immunity.

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