The Cellular Basis for Lack of Antibody Response to Hepatitis B Vaccine In Humans

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Summary

We had previously obtained evidence that among normal subjects the humoral antibody response to hepatitis B surface antigen (HBsAg) was bimodally distributed with about 14% of subjects producing <1,000 estimated radioimmunoassay RIA units. From the study of major histocompatibility complex (MHC) markers in the very poor responders who produced <36 estimated RIA units of antibody, it appeared that there was an excess of homozygotes for two extended haplotypes [HLA-B8, SC01, DR3] and [HLA-B44, FC31, DR7]. This finding suggested that a poor response was inherited as a recessive trait requiring nonresponse genes for HBsAg on both MHC haplotypes and was strengthened by finding a much lower antibody response among prospectively immunized homozygotes for [HLA-B8, SC01, DR3] compared with heterozygotes. In the present study, we have analyzed the cellular basis for nonresponse to this antigen by examining antigen-specific proliferation of T cells from responders and nonresponders in the presence and absence of autologous CD8+ (suppressor) cells.

Peripheral blood cells from nonresponders to HBsAg failed to undergo a proliferative response to recombinant HBsAg in vitro, whereas cells from responders proliferated vigorously. This failure of cells from nonresponders to proliferate was not reversed in cell mixtures containing CD4+ and antigen-presenting cells devoid of CD8+ cells. There was no difference between responders and nonresponders with respect to the number of circulating T cells or their subsets, or the proliferative response to mitogens such as pokeweed or phytohemagglutinin or another antigen, tetanus toxoid.

Our results indicate that our HBsAg nonresponding subjects have a very specific failure in antigen presentation or the stimulation of T helper cells, or both. Our evidence is against specific immune suppression as the basis for their nonresponsiveness. The failure of antigen presentation or T cell help is consistent with recessive inheritance of nonresponsiveness and suggests that response is dominantly inherited.

The humoral antibody response to protein antigens is controlled by class II MHC genes in the mouse and other mammalian species (1, 2). Although antibody responses to synthetic peptides are controlled by MHC genes in mice, experiments to show this in humans are less conclusive (3–5) and the presence or absence of response has not been associated with any specific HLA haplotype. Other studies in vitro using human cells have provided evidence for MHC immune suppression genes to collagen (6), cryptomeria japonica pollen (7), schistosomal antigen (Sj) (8), and streptococcal cell wall (SCW) antigen (9), but comparable animal models have not been found.

The hepatitis B surface antigen is the first protein associated with MHC-linked immune response in both mouse and humans. In the mouse, the antibody response to HBsAg has been shown to be MHC controlled, with certain strains being nonresponders, others mounting an intermediate response, and still other strains a high response (10–14). In general, response is inherited as a dominant trait and failure to respond is inherited as a recessive trait. Studies of the failure to respond to HBsAg among normal Japanese have suggested a high nonresponse rate of >20% and dominant inheritance of the nonresponsiveness marked by the common Japanese MHC haplotype HLA-Bw54, DR4, DRw53 (15). Consistent
with these findings, the same authors have shown that the absence of response could be attributed to suppression, since, in their study, removal of CD8+ cells from nonresponder peripheral blood cells in vitro resulted in proliferative response and antibody production on exposure to HBsAg (15).

In our previous studies, we have noted an increase among Caucasian nonresponders (antibody levels of <36 estimated RIA U/ml) of HLA-DR3 and DR7 and the extended haplotypes [HLA-B8, SC01, DR3] and [HLA-B44, FC31, DR7], and an overrepresentation of homozygotes for the latter two haplotypes (16). This suggested that nonresponsiveness was a recessive trait and, by inference, that the specific immune response to HBsAg is dominantly determined. Such a model does not support a role for T cell suppression. An analysis of the antibody response of ~160 normal Caucasian subjects was consistent with at least two different populations. The more poorly responding population comprised ~14% of the total (17). To test whether [HLA-B8, SC01, DR3] marked a recessively expressed nonresponse gene for HBsAg, we prospectively immunized homozygotes and heterozygotes for the haplotype. The homozygotes were, in general, non- or hyporesponders and had significantly lower antibody levels than the heterozygotes (17). In the present study, we analyze the cellular mechanism of nonresponse to HBsAg, particularly with respect to a possible role for immune suppression.

Materials and Methods

**Subjects.** Normal healthy Caucasian volunteers who had previously received a full course of intramuscular (deltoid) immunization with Heptavax-B (Merck Institute for Therapeutic Research, West Point, PA) were studied. Three subjects were responders and had mean antibody levels >2,000 estimated RIA U/ml as determined by RIA (AUSAB; Abbott Laboratories, North Chicago, IL) whereas four were nonresponders and had antibody levels <36 estimated RIA U/ml 8 wk after the last injection of vaccine. All subjects were <40 yr-of-age and in good health; all were typed for HLA types [HLA-B8, SC01, DR3] and [HLA-B44, FC31, DR7], and C4A, and C4B by standard methods (16). This suggested that nonresponsiveness was a recessive trait and, by inference, that the specific immune response to HBsAg is dominantly determined. Such a model does not support a role for T cell suppression. An analysis of the antibody response of ~160 normal Caucasian subjects was consistent with at least two different populations. The more poorly responding population comprised ~14% of the total (17). To test whether [HLA-B8, SC01, DR3] marked a recessively expressed nonresponse gene for HBsAg, we prospectively immunized homozygotes and heterozygotes for the haplotype. The homozygotes were, in general, non- or hyporesponders and had significantly lower antibody levels than the heterozygotes (17). In the present study, we analyze the cellular mechanism of nonresponse to HBsAg, particularly with respect to a possible role for immune suppression.

**Isolation and Enrichment of T Cell Subsets.** T cells were enriched by E-rosetting (20). CD4+ T cells and CD8+ T cells were enriched by a panning method as described earlier (21-23) and were cultured in RPMI 1640 supplemented with 10% FCS, 2 mmol/liter of t-glutamine, 50 U/ml of penicillin, and 50 μg/ml of streptomycin. Fresh cells were used in all kinetic and reconstitution experiments. Immunofluorescence and dose-response experiments were carried out with thawed frozen cells.

**Isolation of Adherent Cells.** Monocytes were collected from adherent fractions and were >96% Mo-1+ and <3% CD3+, as determined by FACS® (Becton Dickinson & Co., Mountain View, CA) analysis (24). Indirect immunofluorescence on 106 cells was done using anti-CD3, anti-CD4, and anti-CD8 from Becton Dickinson and Co. (Mountain View, CA) and FITC-conjugated goat anti-Fab antibody (25). Cells were analyzed on an Epic 750 Series cell sorter (Coulter Electronics Inc., Hialeah, FL) coupled with an Inova 90 5-W argon laser (Coherent Inc., Palo Alto, CA). Direct immunofluorescence was analyzed with PE-conjugated anti-CD29 (4B4) and anti-CD45R (2H4) antibodies (Coulter Electronics Inc.). The proportion of different cells was given as percent (26).

**Antigen-Specific T Cell Proliferation.** Unfractionated PBL (106/ml) were incubated with different concentrations of HBsAg (0.01, 0.05, 0.1, 0.2, 0.5, 1, 10, and 20 μg/ml). Tetanus toxoid at 10 μg/ml and PHA and PWM at 0.5 μg/ml were used as controls. [H]Thymidine incorporation was measured after 3, 5, 7, and 9 d in culture.

### Table 1. HLA Types of Nonresponders and Responders to Hepatitis B Vaccine

| Individuals* | HLA types |
|--------------|-----------|
| **Nonresponders** |  |
| 1 A2, A28, Cw2, B44, BX, SC31, SC33, DR2, DRX, DQw1, DQwX |  |
| 2 A1, B8, SC01, DR3, DQw2/A1, Cw3, Bw60, SB42, DR4, DQw3 |  |
| 3 A1, B8, SC01, DR3, DQw2/A1, B18, SC01, DR3, DQw2 |  |
| 4 A1, B8, SC01, DR3, DQw2/A1, B8, SC01, DR3, DQw2 |  |
| **Responders** |  |
| 1 A25, A32, B44, B21, Cw5, SC31, DRw12, DR7, DQw2, DQw3 |  |
| 2 A1, A23, B7, B38, Cw7, SC31, DR2, DR4, DQw1, DQw3 |  |
| 3 A1, A2, B44, Bw60, Cw3, SCFC31, DR7, DQw2 |  |

* Three of four nonresponders were genotyped (*italics*), all other subjects were phenotyped.
Because of the well-known variability in background [\( ^{3}\text{H} \)] thymidine incorporation from experiment to experiment and from individual to individual, a stimulation index (SI) was calculated for each mitogen for each set of experiments. The SI is the ratio of experimental to background net counts per minute. Background and specific antigen and mitogen experiments were always done simultaneously for any one subject, although, in general, at least one responder and one nonresponder were studied together.

Reconstitution Experiments. The three fractionated cell populations, obtained as described, were mixed (0.5 \( \times 10^{6} \) CD4\(^+ \) T cells, 0.5 \( \times 10^{6} \) CD8\(^+ \) T cells, and 2 \( \times 10^{6} \) monocytes in NBCS medium) and cultured. For the definitive experiments, concentration of HBsAg was 0.2 \( \mu \text{g/ml} \) and [\( ^{3}\text{H} \)] thymidine incorporation was measured up to day 7 in culture.

Statistical Analysis. All cultures were done in triplicate and the proliferation data were determined as the arithmetic means. Results were expressed as the difference in the cpm of the stimulated and unstimulated cultures +/- SEM, as \( \Delta \) of cpm \( \pm \) SEM of cpm or as the mean \( \pm \) one SD and converted to SI, as above.

Results

Proliferation in the Presence of HBsAg. The optimal dose of HBsAg for proliferation experiments was determined using cells from responders. One such experiment is shown in Fig. 1 where the optimal HBsAg concentration was 0.2 \( \mu \text{g/ml} \). Higher concentrations elicited less proliferation and no proliferation was observed at concentrations of HBsAg of 1 \( \mu \text{g/ml} \) or higher. Cells from four nonvaccinated healthy controls or from the three vaccinated but nonresponding individuals did not show proliferation under any conditions tested.

Kinetics of Proliferative Responses to HBsAg, Tetanus Toxoid, and Lectins (PHA and PWM). These experiments, shown in Table 2 and Fig. 2, were performed on freshly isolated lymphocytes from three responders and three nonresponders. As expected, cells from both the responders and nonresponders showed significant proliferation on exposure to tetanus toxoid, PHA, and PWM. Fig. 2 displays the peak proliferative re-

![Figure 1. Proliferative response to different concentrations of HBsAg. Data are expressed as cpm \( \pm \) SE. 10\(^{6} \) PBL were incubated in different concentrations of HBsAg in a final volume of 200 \( \mu \text{l} \) in triplicate. After 6 d of incubation, 0.8 \( \mu \text{Ci of} \) [\( ^{3}\text{H} \)] Tdr in 20 \( \mu \text{l} \) was added to each culture and kept overnight (18 h). (○) One responder; (□) one nonresponder; (□) a nonvaccinated individual.]

| HBsAg (μg/ml) | SI* | None | PHA | PWM |
|---------------|-----|------|-----|-----|
| 0             | 0.5 | 0.5  | 0.5 | 0.5 |
| 0.2           | 1   | 10   | 10  | 10  |
| 0.5           | 2   | 20   | 20  | 20  |
| 1             | 4   | 40   | 40  | 40  |
| 5             | 20  | 200  | 200 | 200 |

Table 2. Optimal Proliferative Response of Lymphocytes to HBsAg, Tetanus Toxoid, PHA, and PWM in HBsAg-Vaccinated Individuals.

Results are expressed as mean cpm \( \pm \) 1 SD of triplicate cultures, and are the result at peak time for each antigen or stimulator.

SI: Stimulation index = mean cpm in cultures of cells with HBsAg/mean cpm in cultures of cells without HBsAg.
response to HBsAg, PHA, and PWM in each subject. Although there was considerable variability in the proliferative response to this antigen and these mitogens, there was no pattern and no correlation with proliferation in response to HBsAg in the same subjects. The optimal response to tetanus toxoid was between days 5 and 7, whereas the greatest responses to PHA and PWM occurred on days 3 and 5. The responses to PHA and PWM were significant even on day 7. These results contrasted with the proliferative response to HBsAg, in which responders showed peak proliferation on day 7, ranging from 39,000 to 57,000 cpm (Fig. 2 A). Maximum values for nonresponders fell between -130 and 210, not different from control cultures without HBsAg when the SEM of Δ cpm was taken into account (Fig. 2 B). The lack of specific proliferative response to HBsAg by cells from nonresponders is evident over the entire 9-d period of observation.

Absence of Specific T Cell Proliferation to HBsAg in Nonresponders is not Mediated by CD8+ Cells. Enriched CD4+ cells (85–95% pure) and monocytes (96% pure) were cocultured with and without HBsAg (0.2 μg/ml) for 7 d. The results are shown in Table 3. For all responders, culturing reconstituted monocytes with CD4+ cells and antigen resulted in significant proliferation (from 3,170 to 42,570 cpm, or 3.3 to 18.8 times greater than background). Cells from nonresponders, including one subject tested twice, showed proliferation values in response to HBsAg comparable to background cultures (0.89–1.34 times background). Thus, removal of CD8+ cells did not change the lack of proliferative response to HBsAg by cells from nonresponders.

Reconstitution experiments were also performed using tetanus toxoid and PHA as control antigens. Table 4 shows the results of these experiments. PHA (not shown) and tetanus toxoid produced significant proliferation in cells from both responders and nonresponders. Reconstituted monocytes and CD4+ cells, depleted of CD8+ cells, from nonresponders failed to proliferate in response to HBsAg in all instances. The addition of CD8+ cells (58–69% pure) to the above cell mixtures resulted in decreased proliferative responses to tetanus toxoid in six of seven individuals. With PHA (results not shown) this phenomenon was evident in only three of seven subjects. Addition of CD8+ cells to cultures with HBsAg produced even lower values in one of three responders and in four of four nonresponders; and in three of five experiments in nonresponders, proliferation was below the background level, indicating that the CD8+ cells had suppressor function (Table 4).

Table 3. Antibody Response and Proliferative Response of CD4+ T Lymphocytes to HBsAg

| Individuals | Antibody response* | HBsAg Added to Monocytes and CD4+ T cells† |
|-------------|--------------------|--------------------------------------------|
|             | Pre-booster | Post-booster | None† | 0.2 μg/ml§ | SI† |
| Nonresponders | 1 | 32 | 2,991† | 1,491 ± 140 | 1,334 ± 139 | 0.9 |
|              | 2 | 8 | 20 | 6,576 ± 2,116 | 7,366 ± 1,264 | 1.1 |
|              | 3a | <8 | <8 | 2,455 ± 325 | 2,824 ± 642 | 1.1 |
|              | 3b | <8 | <8 | 874 ± 644 | 1,174 ± 530 | 1.3 |
|              | 4 | <8 | <8 | 1,226 ± 172 | 1,235 ± 243 | 1.0 |
| Responders | 1 | 456 | 59,840 | 1,554 ± 152 | 5,137 ± 495 | 3.3 |
|              | 2 | 8,068 | 86,300 | 2,268 ± 396 | 42,570 ± 2,835 | 18.8 |
|              | 3 | 52,267 | 244,480 | 205 ± 121 | 3,172 ± 911 | 15.5 |

* Antibody levels as RIA U/ml were measured in serum obtained either before or 4–6 wk after a booster challenge with recombinant HBsAg vaccine.
† The cells used in these studies were obtained 8 wk after a full vaccination course with recombinant HBsAg vaccine.
§ Data expressed as mean cpm of triplicate cultures ± SD.
‖ SI = mean cpm in cultures of cells with HBsAg/mean cpm in cultures of cells without HBsAg.
¶ This subject was considered a nonresponder based on an antibody level of <36 RIA U/ml after a standard course of three injections.
Numbers of Circulating CD4+ and CD8+ T Cells in Responders and Nonresponders to HBsAg. As can be seen from Table 5, there was no difference in the numbers of total T cells (CD3+), helper T cells (CD4+), or suppressor T cells (CD8+) in nonresponders to HBsAg compared with responders. There was similarly no difference in the numbers of CD29 (4B4+) and CD45R (2H4+) subsets of CD4+ cells.

Discussion

The failure to mount a humoral immune response to a defined antigen can occur by one or more of a number of possible MHC-determined mechanisms. There can be a hole in the T cell repertoire determined by specific MHC molecules during thymic education of T cells (1–5, 27–29) or there can be a failure of class II molecules of the MHC to interact with processed antigen with resultant failure to activate T helper (CD4+) cells. Either of these mechanisms can be looked upon as a failure in antigen presentation and would be expected to be manifested only in those individuals in whom there is a defect specified by both chromosomes. Thus, nonresponders would be homozygous for nonresponder MHC genes and nonresponsiveness would be inherited as a recessive trait. On the other hand, if the failure to respond is due to the presence of antigen-specific suppressor (CD8+) cells, this would be expected to require only one chromosome specifying suppression, nonresponders would generally be heterozygotes for immune suppressor genes and nonresponse would thus be inherited as a dominant trait.

All studies of the antibody response to a standard course of immunization with HBsAg have demonstrated that from 4 to 10% of normal healthy subjects do not respond or respond poorly (16, 30–34). The exact percentage depends to some extent upon the definition of non- or hyporesponse. The bimodality of the antibody response of a large normal population of Caucasians (17) is compatible with genetic heterogeneity in the determination of the immune response to HBsAg. That the MHC is involved in at least one genetic determinant was supported by the finding that HLADR7 (35), DR3, DR4, and DR7 (16, 17, 36), and the two extended haplotypes [HLA-B8, SC01, DR3] and [HLA-B44, FC31, DR7] (16, 17) were increased among the Caucasian nonresponders. Since homozygotes for these alleles and haplotypes appeared to be particularly frequent among the nonresponders, it appeared that the MHC was contributing as a recessive trait (16). This hypothesis was tested by the prospective immunization of homozygotes and heterozygotes for [HLA-B8, SC01, DR3]. The homozygotes produced significantly less antibody than the heterozygotes (17), consistent with there being a recessively inherited and expressed nonresponse gene for HBsAg on [HLA-B8, SC01, DR3]. In our current study, we were unable to establish a reproducible antibody synthesis assay system and therefore chose to use proliferation as a reliable index of the secondary T cell response. Our findings are in all respects different from those of Watanabe et al. (15) who found that the absence of a proliferative response and antibody synthesis by peripheral blood cells.
of nonresponders in vitro could be reversed by removal of CD8+ cells. Watanabe et al. (15) also noted that many of their nonresponding subjects carried the haplotype HLA-Bw54, DR4, DRw53, common in Japanese, as heterozygotes. After making some assumptions based on linkage disequilibrium, these authors found that, by the Thomson-Bodmer analysis (37), inheritance of the MHC markers was most likely dominant (15). The marked differences in findings between the Japanese studies and ours may derive from ethnic differences in the populations studied. This possibility is enhanced by the observations of a second group in Japan who also found a major role for suppressor cells in unresponsiveness to HBsAg (38). Another possible source of discrepancy is that our subjects were immunized intramuscularly into the deltoid, the route reported to produce optimal immunization (39), whereas Watanabe et al. (15) subjects were vaccinated subcutaneously, known to be suboptimal (40).

There have been many elegant studies of the antibody and T cell proliferative responses to HBsAg in inbred mice. Some strains produce no detectable antibody as a result of homozygosity for nonresponding H-2 haplotypes such as H-2d or H-2k (10-14). Thus, in the mouse in general, nonresponsiveness to HBsAg is a recessive trait and response is dominant, since the F1 offspring of responder x nonresponder crosses are responders (10-14). There is a role for suppressor cells in the murine anti-HBs response, albeit not as an explanation for nonresponse. In the low responder B10.BR strain, depletion of CD8+ (Lyt-1,2+) (suppressor) T cells increased both the subtype and group specific anti-HBs responses. However, this depletion did not correct the T cell proliferative defect characteristic of the strain. Predictably, T cell proliferation in the responder mice was mediated by antigen-presenting cells, and HBsAg-specific T cell proliferation was mapped to the I-A and I-E subregions of the mouse H-2 region. These studies identified the defect in the HBsAg nonresponders as being at the level of T cell-APC interaction (10-14, 38).

Mechanisms for the immune response to HBsAg or its failure in humans are less clear. Small numbers of HBsAg-specific T and B cells are present in vaccinated individuals (41-44). In one study, T helper clones specific for HBsAg proliferated in vitro upon stimulation with the antigen (43). Moreover, antibodies specific for the antigen enhanced cell proliferation, suggesting antibody regulation of T cells specific for the same antigen (44). However, it could not be concluded that the lack of response in nonresponders resulted from the lack of antibodies. In other reports, peripheral blood cells from hyperimmunized individuals synthesized anti-HBs in vitro, although there was no cell-mediated hypersensitivity or T cell proliferative response to HBsAg (45, 46). In another study, nonresponders to HBsAg had significantly higher absolute and relative numbers of CD2+ (T11+, CD57+ (HNK-1+), and CD8+ (T8+) lymphocytes and increased numbers of suppressor lymphocytes (47). Cell coculture experiments suggested that the early stage of nonresponsiveness is mainly due to a defect in the B cell repertoire, while the later stage is caused by HBsAg-specific suppressor T cells (48).

Responsiveness and nonresponsiveness to a number of antigens has been investigated by Sasazuki and coworkers. They found that HLA-DR molecules restrict the interaction of CD4+ helper T cells and APC in studies of the response to SCW (9, 49-51), SJ (8), and Mycobacterium leprae antigen (ML) (52). In these studies, the addition of anti-HLA-DR mAbs completely abolished the immune response in vitro to these antigens in immune subjects (9, 49-51). The same group showed that responses to these antigens were recessive traits, whereas nonresponsiveness was dominantly expressed and associated with different HLA antigens (15, 50-53). In the mouse, the response to SCW was in association with HLA-DR2, DR5, and DQw1, the response to SJ with HLA-Dw12, and to ML with HLA-B35, Dw2 (50, 51, 53). The IgE response to cedar pollen was found to be negatively associated with HLA-DQw3. The lack of response to the hepatitis B vaccine was found to be mediated by CD8+ cells in HLA-DQw4 positive individuals (15, 49, 51, 53, 54).

Only further work will resolve the differences reported.

### Table 5. T Cell Subpopulation Proportions of Nonresponders and Responders to HBsAg

| Individuals | CD3+ | CD4+ | CD8+ | CD4+/CD8+ | Percent of CD4+ T Cells* |
|-------------|------|------|------|-----------|-------------------------|
|             |      |      |      |           | CD29 (4B4+) | CD45R (2H4+) |
| Nonresponders | 66   | 38   | 24   | 1.58      | 46          | 46          |
| 2           | 79   | 44   | 29   | 1.51      | 65          | 37          |
| 3           | 76   | 53   | 17   | 3.11      | 65          | 37          |
| 4           | ND   | 59   | 25   | 2.36      | 59          | 50          |
| Responders  | 70   | 49   | 23   | 2.13      | 55          | 44          |
| 2           | 62   | 46   | 10   | 4.60      | 60          | 46          |
| 3           | 67   | 47   | 29   | 1.60      | 52          | 47          |

* The percentages of CD29 and CD45R subsets of CD4+ were obtained by immunofluorescence using specific mAbs and the Epic 750 cell sorter (Coulter Electronics Inc.).
by different groups. Our results with respect to the response or nonresponse to HBsAg are consistent with the results of our earlier studies (16, 17) and those of Milich and coworkers (10–14) in the mouse. They are further supported by preliminary data from family studies (our unpublished results) in which we find direct evidence for dominant inheritance of responsiveness and recessive inheritance of nonresponsiveness.

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