ENHANCEMENT OF THE IMMUNE RESPONSE TO HEPATITIS B SURFACE ANTIGEN

In Vivo Administration of Antiidiotype Induces Anti-HBs That Expresses a Similar Idiotype

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Immunoglobulin idiotypes (Id)[1] have been studied extensively since they were first recognized by Kunkel et al. (1) and by Oudin and Michel (2) in 1963. Id, along with homologous anti-Id antibodies, are thought to be components of a network of complex reactions that regulates a given immune response. Such regulation at the level of Id recognition was postulated by Jerne (3) to involve a series of Id-anti-Id reactions that are able to control immune responses at both the afferent and efferent modes. Numerous studies have implicated Id networks in regulating the immune response to a large variety of haptens and protein or carbohydrate antigens (reviewed in 4 and 5). Recently, anti-Id antibodies have been used to enhance the murine antibody response to transplantation antigens (6, 7) and to induce protective immunity in mice against lethal infection with trypanosomiasis (8).

Hepatitis B surface antigen (HBsAg), the envelope material of hepatitis B virus (HBV), is produced in an overwhelming excess by infected hepatocytes during the course of an HBV infection (9, 10). The presence of HBsAg is often associated with the development of chronic liver disease and primary carcinoma of the liver (11). Previous studies from this laboratory (12–15) have characterized a common Id shared by human antibodies to HBsAg (anti-HBs). This common Id was also detected on anti-HBs produced in BALB/c mice and six other species, indicating that an interspecies idiotypic cross-reaction was expressed (16). The involvement of an Id-anti-Id network was indicated in that the numbers of anti-HBs plaque-forming units (PFU) in the spleens of BALB/c mice were enhanced by prior injection of anti-Id antibodies (17).

In this report, we explore the effects of in vivo administration of anti-Id antibodies on the humoral murine anti-HBs response. The serologic characteristics of the anti-Id–induced anti-HBs molecules were examined for expression

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[1] *Abbreviations used in this paper*: Anti-HBs, antibodies to hepatitis B surface antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; Id, idiotype; PFU, plaque-forming unit; RIA, radioimmunoassay; SPRIA, solid-phase radioimmunoassay.

of the interspecies Id markers. The implications of these observations with regard to Id networks and HBV infections are discussed.

Materials and Methods

Preparation and Purification of Anti-Id Antibodies. The preparation of rabbit anti-Id antiserum and its specificity have been described in detail elsewhere (12, 13). Anti-Id antisera were affinity purified by acid elution from Id-containing immunoadsorbent columns by methods similar to those reported elsewhere (18). Concentrations of anti-Id antibodies were determined spectrophotometrically using an extinction coefficient of 15 for a 1% solution at 280 nm.

Determination of Anti-HBs Activity. The anti-HBs activity in the mouse antisera was determined by a solid-phase radioimmunoassay (RIA) referred to as micro-SPRIA (19). Briefly, 200 ng of HBsAg, subtype *adw*, was coated onto the wells of a microtiter plate. Fivefold dilutions of mouse antisera were added to the wells after postcoating and washing steps. The unbound serum protein was washed off and ^{125}I-labeled goat anti-mouse γ chain-specific antiserum (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) was added. Residual radioactivity was removed and the wells were washed and counted. The anti-HBs endpoint titers were determined as the dilution of mouse antisera that gave an arbitrary positive (S) to negative (N) cpm ratio of 2.1 (19). IgM anti-HBs was similarly detected using a radiolabeled rabbit anti-mouse μ chain-specific antiserum that was generated in this laboratory. This reagent was prepared by adsorption of a rabbit anti-mouse monoclonal IgM antiserum over an immunoadsorbent column containing mouse IgG to remove anti-light chain and anti-heavy chain isotype activities. The immunization protocols to produce the reagent and the preparation of immunoadsorbent columns have been described in detail elsewhere (18, 20). In addition, 200 ng of HBsAg, subtypes *ayw* and *adr*, were coated on the wells of a microtiter plate, and a micro-SPRIA was similarly performed to ascertain the subtype specificity of the mouse anti-HBs. A commercial solid-phase RIA kit was also used for quantification of anti-HBs (AUSAB; Abbott Laboratories, North Chicago, IL).

Expression of the Interspecies Id on Anti-HBs. The detection of the interspecies Id on mouse anti-HBs has been described previously (16). Briefly, a limiting dilution of rabbit anti-Id antisera was coated onto the wells of microtiter plates, and the ability of mouse antisera to block the binding of ^{125}I-labeled Id determined. The percentage inhibition of binding was calculated as previously described (12).

Isolation and Purification of HBsAg. The purification of 22-nm HBsAg particles, subtypes *adw*, *ayw*, and *adr*, was performed by the method described elsewhere (21). Human plasma positive for the three HBsAg subtypes were kindly provided by Dr. H. Fields, Centers for Disease Control, Atlanta, GA. The concentration of HBsAg was determined using an extinction coefficient of 37.26 for a 1% preparation at 280 nm.

In Vivo Administration of Anti-Id Antibodies. Adult female BALB/c mice (Charles River Breeding Laboratories, Wilmington, MA) were given intraperitoneal injections of affinity-purified anti-Id antibodies either in saline or as an alum precipitate at pH 7.0 (22). Control antibodies were IgG preparations from normal serum obtained from rabbits before injection of the Id (pre-IgG). These antibodies were also given in soluble or alum-precipitated forms, and the concentration of IgG was similarly determined using an extinction coefficient of 15. Groups of mice received either anti-Id or pre-IgG before an intraperitoneal injection of 6 μg of HBsAg in saline. Mice were bled 12 d after the injection of HBsAg. Serum obtained from each mouse before any immunization served as a negative control for each animal. Based on the level of anti-HBs activity of each group of mice, the optimal dose of anti-Id antibodies and the optimal time interval between injections of anti-Id and HBsAg were established. These parameters were then used to induce anti-HBs activity by the injection of anti-Id antibodies alone.

Iodination. 50 μg each of goat anti-mouse γ chain, rabbit anti-mouse μ chain, and the anti-HBs Id preparations were labeled with Na^{125}I (Amersham Corp., Arlington Heights, IL) using the chloramine-T reaction (23). Protein-associated and free ^{125}I were separated

on PD-10 columns (Pharmacia Fine Chemicals, Piscataway, NJ). More than 95% of the radioactivity of the labeled preparations was precipitable with a final concentration of 10% trichloroacetic acid.

Statistical Methods. The levels of significance (*P* values) were obtained using the two-tailed Student's *t* test (24) on the $\log_{10}$ of the reciprocal arithmetic mean anti-HBs titer of each group of mice.

Results and Discussion

In a recent study (17), we reported that in vivo administration of anti-Id antibodies before the injection of HBsAg increased the number of anti-HBs PFU in the spleens of BALB/c mice when compared with mice injected with control pre-IgG before HBsAg stimulation (17). In addition, mice receiving anti-Id antibodies alone had a significantly higher number of anti-HBs PFU when compared with mice treated with only control antibodies. These data suggested that the immune response to HBsAg may be regulated via Id-anti-Id interactions. In this report, the presence of anti-Id–induced anti-HBs activity in serum of BALB/c mice was investigated to ascertain whether these antibodies expressed an interspecies Id that would be indicative of an Id-anti-Id–controlled network.

The first experiment was performed to determine the most immunogenic form(s) of anti-Id antibodies for enhancing the anti-HBs response. Our previous study used soluble anti-Id antibodies in the modulation of the murine anti-HBs response at the spleen cell level; however, other investigators demonstrated that high levels of antibody could be induced in their Id systems without antigen stimulation, by injecting cross-linked anti-Id (25, 26). Because higher titers of anti-HBs were generated in experimental animals when HBsAg was alum-precipitated rather than in aqueous solution (22), we decided to compare the effects on the anti-HBs response by injecting alum-precipitated material vs. soluble anti-Id antibodies before HBsAg stimulation. As shown in Table I, the mean anti-HBs titer was higher in groups of mice receiving alum-precipitated anti-Id when compared with anti-Id in saline before HBsAg injection (AUSAB titers of 487.5

TABLE I

*Comparison of Alum-precipitated and Soluble Anti-Id for Induction of Anti-HBs**

First injection	Second injection	No. of mice	Anti-HBs response (mean ± SEM)‡	Micro-SPRIA	
				Anti-HBs IgG§	Anti-HBs IgM‖
Anti-Id alum-precipitated	HBsAg	4	487.5 ± 315.0	4,938	1,250
Anti-Id soluble	HBsAg	4	72.5 ± 50.0	86	1,000
Pre-rabbit IgG alum-precipitated	HBsAg	4	<5.0¶	<5.0	<5.0
Pre-rabbit IgG soluble	HBsAg	4	6.25 ± 1.08	30	50

* Each group of mice received 40 μg of anti-Id or pre-IgG on day 0, followed by 6 μg of HBsAg on day 14, all by the intraperitoneal route. Mice were bled on day 26.

‡ The values are the reciprocal dilution of antisera that gave an endpoint S/N of 2.1 (see Materials and Methods) as measured by AUSAB.

§ The mean value of reciprocal dilution of antisera that gave an endpoint S/N of 2.1 as measured by SPRIA using ^{125}I-labeled goat anti-mouse γ chain-specific antiserum.

‖ The mean value of reciprocal dilution of antisera that gave an endpoint S/N of 2.1 as measured by SPRIA using ^{125}I-labeled rabbit anti-mouse μ chain-specific antiserum.

¶ All mice were negative for anti-HBs at a serum dilution of 1:5.

vs. 72.5). Only one of four mice produced a detectable anti-HBs response in the soluble control pre-IgG group, whereas no mice receiving alum-precipitated pre-IgG before HBsAg produced a detectable anti-HBs response at a 1:5 serum dilution (Table I). In this experiment, the anti-HBs response was measured by both a commercial RIA that detects IgM and IgG anti-HBs and a solid-phase RIA. For the remaining experiments, the solid-phase RIA was used for titration of the mouse antisera because this test is more sensitive, less expensive, and requires lower quantities of serum (50 vs. 200 μl) when compared with the AUSAB (19). It was noteworthy that primarily IgM anti-HBs was detected by an IgM type-specific RIA in the group of mice injected with the soluble anti-Id preparation. This was similar to our previous observations (17) where the number of direct IgM anti-HBs PFU was enhanced by prior injection of soluble anti-Id.

In the second set of experiments, the optimal time interval between a primary injection of alum-precipitated anti-Id antibodies and a subsequent HBsAg inoculum for enhancing the anti-HBs response was determined. Only IgG anti-HBs was measured since alum-precipitated anti-Id generated a potent secondary IgG response to HBsAg. In the previous experiment, the alum-precipitated anti-Id–treated mice generated a fourfold higher IgG anti-HBs titer when compared with IgM (Table I). Also, previous observations in reference to the secondary humoral anti-HBs response indicated that IgG was the predominant antibody class present between 10 and 20 d after HBsAg boost (27). For these reasons, serum was routinely obtained 12 d after HBsAg injection and only IgG anti-HBs was assayed in the remainder of the study. Based on the data presented in Table II, the optimal anti-HBs response occurred when HBsAg was given 14 d after priming with anti-Id. In each instance, groups of mice that received anti-Id antibodies before HBsAg generated a higher anti-HBs response when compared with the mice given pre-IgG. The extremely high standard errors of the mean shown in Table II resulted from both the small sample size ($n = 4$) and the individual variations in anti-HBs titers among mice.

The optimal quantity of anti-Id required for enhancement of the anti-HBs response was determined in the next experiment (Table III). Previous reports (28, 29, reviewed in 30) have indicated that the concentration of anti-Id antibodies was important in modulating the immune response to various antigens. Based on the two-tailed Student's t test, the greatest significant difference ($P < 0.001$) of the anti-HBs titer was noted in that group of mice inoculated with 50 μg of alum-precipitated anti-Id vs. pre-IgG before HBsAg stimulation. Significant differences were also obtained in the groups of mice injected with 5 and 200 μg of anti-Id when compared with control groups inoculated with similar doses of pre-IgG. Although the mean anti-HBs titer was higher in the group treated with 50 μg of anti-Id when compared with the groups given 5 or 200 μg of anti-Id, the difference was not statistically significant. The reasons for this higher mean anti-HBs titer again may have reflected the small sample size ($n = 4$) and the individual variation of mice. No significant difference was noted in the anti-HBs response between the groups of mice receiving 500 ng of either anti-Id or pre-IgG before HBsAg ($P > 0.2$). Also, the mean anti-HBs titer was lower when compared with groups of mice receiving higher quantities of anti-Id. These findings are similar to results previously reported in other Id systems where low

TABLE II

*Optimal Time Interval Between Injection of Anti-Id and HBsAg for Induction of Anti-HBs**

First injection	HBsAg injection (No. of days after primary inoculation)	Anti-HBs response‡ (mean ± SEM)
Anti-Id	7	130 ± 32.1
	14	4,222 ± 1,825.7
	21	200 ± 64.6
Pre-IgG	7	5 ± 5.0
	14	<5.0§
	21	30 ± 11.5

* Each group of four mice received 40 μg of alum-precipitated anti-Id or pre-IgG on day 0, followed by 6 μg of HBsAg on the days specified, all by the intraperitoneal route. Mice were bled 12 d after injection with HBsAg.

‡ The values are the reciprocal dilution of antiserum that gave an endpoint S/N of 2.1 (see Materials and Methods) as measured by the IgG anti-HBs SPRIA.

§ All mice were negative for anti-HBs at a serum dilution of 1:5.

TABLE III

*Effect of Dose of Alum-precipitated Anti-Id for Induction of IgG Anti-HBs Using Micro-SPRIA**

First injection	Second injection	Reciprocal arithmetic mean titer	Log_{10} of the reciprocal arithmetic mean titer	Standard deviation‡	
500 ng anti-Id§	HBsAg	583	2.76	0.77	$(P > 0.2)$‖
500 ng pre-IgG	HBsAg	86	1.93	0.26	
5 μg anti-Id	HBsAg	4,938	3.69	0.32	$(P < 0.02)$
5 μg pre-IgG	HBsAg	66	1.82	0.88	
50 μg anti-Id	HBsAg	9,378	3.97	0.10	$(P < 0.001)$
50 μg pre-IgG	HBsAg	6	0.78	0.18	
200 μg anti-Id	HBsAg	4,344	3.64	0.62	$(P < 0.01)$
200 μg pre-IgG	HBsAg	30	1.48	0.25	

* Each group of four mice received various concentrations of alum-precipitated antibodies on day 0, followed by 6 μg of HBsAg on day 14. Mice were bled on day 26.

‡ The standard deviation of the log_{10} of the reciprocal arithmetic mean titer.

§ Several of the mouse antisera were negative at the dilution tested. To facilitate the computations, the reciprocal of the titer was considered 0.5, assuming that greater than twofold concentration of the sample would give a positive result.

‖ Determined by the two-tailed Student's *t* test.

amounts of anti-Id antibodies failed to modulate the immune response (28, 29). Due to the heterogeneity of the variances among the groups receiving different antibody concentrations before antigenic stimulation, the reciprocal arithmetic mean anti-HBs titers were expressed as values to $\log_{10}$ to facilitate the use of the parametric Student's t test rather than a nonparametric test (i.e., Kruskal-Wallis) for the calculations of significance (17). It is noteworthy that some mice that received pre-IgG before HBsAg produced an IgG anti-HBs response (Table III). The reasons for such a response are not known. However, it was consistent throughout the different experiments among the individual groups treated with pre-IgG. Based on the data presented in Table III, 50 μg of anti-Id was selected as the optimal dose for generating anti-HBs in further experiments.

Previously (17) we demonstrated that BALB/c mice receiving anti-Id antibodies alone generated indirect IgG anti-HBs PFU. These data indicated that anti-Id antibodies could induce an anti-HBs response without HBsAg stimulation. Similar results (6, 25, reviewed in 30) have been reported in other Id systems where administration of anti-Id without antigen exposure produced idiotype-bearing molecules with antigen-binding activity. Based on the information established from the above experiments (Tables I–III), we attempted to induce anti-HBs in the serum of BALB/c mice by injecting anti-Id alone. A statistically significant IgG anti-HBs response was generated in mice receiving two injections of 50 μg of alum-precipitated anti-Id when compared with mice given similar injections of pre-IgG ($P < 0.001$) (Table IV). A mean anti-HBs titer of 1:1,000 was obtained with four mice receiving anti-Id. Conversely, no anti-HBs was detected in four mice injected with control pre-IgG at a 1:5 serum dilution. These data indicate that anti-Id alone can produce detectable humoral anti-HBs in BALB/c mice. Analysis of the anti-Id–induced anti-HBs demonstrated the expression of the interspecies Id (Table IV). In this regard, four sera containing anti-HBs generated by anti-Id induction inhibited the Id-anti-Id reaction from 38 to 54%, whereas <10% inhibition was obtained with the non–anti-HBs-

TABLE IV

*Induction of IgG Anti-HBs Antibodies that Express the Idiotype by Injection of Anti-Id**

First injection	Second injection	Reciprocal arithmetic mean titer	Log_{10} of the reciprocal arithmetic mean titer	Standard deviation‡	Percent inhibition of binding of the Id-anti-Id reactions§
Anti-Id	Anti-Id	1,000	3.0	0.24 ($P < 0.001$)¶	38–54‖
Pre-IgG**	Pre-IgG	<5	0.3	0.00	0–9

* Each group of four mice received two injections of either 50 μg of alum-precipitated anti-Id or pre-IgG antibodies, 14 d apart. Mice were bled 12 d after the second injection.

‡ The standard deviation of the $\log_{10}$ of the reciprocal arithmetic mean titer. Four mice were injected in each group.

§ All sera were tested for the ability to inhibit the Id-anti-Id reaction at a 1:10 dilution (16).

‖ The range of mean values obtained from triplicate samples.

¶ Determined by the two-tailed Student's t test.

** All four mouse antisera were negative at the dilution tested. To facilitate the computations, the reciprocal of the titer was considered to be 0.5, assuming that greater than twofold concentration of the sample would give a positive result.

containing sera from the four mice injected with pre-IgG. In addition, all four preimmune sera from the mice that produced anti-HBs by anti-Id injection inhibited the binding of the Id to its anti-Id antiserum by <10% (unpublished data). Together, these data indicate that in vivo administration of anti-Id antibodies in BALB/c mice induced anti-HBs that expressed a similar interspecies Id. This interspecies Id was also detected in anti-HBs-positive mice that received either anti-Id or pre-IgG before HBsAg (Table V).

It was of interest that anti-HBs from the four mice receiving anti-Id before HBsAg inhibited the Id-anti-Id reaction to a greater degree (32–61%) when compared with anti-HBs from two of the four mice given pre-IgG before HBsAg. Although this greater amount of inhibition of the Id-anti-Id reaction may have reflected higher concentrations of anti-HBs in the mice treated with anti-Id, previous studies indicated that the level of interspecies Id inhibition was not related to the anti-HBs titer (16). The two mice that received pre-IgG before HBsAg and failed to produce a detectable anti-HBs response inhibited the Id-anti-Id reaction by <5%.

Serologically, HBsAg contains a group-common antigenic reactivity referred to as the *a* determinant and two sets of allelic subtype determinants *d* or *y* and *w* or *r* (30, 31). In this regard, four possible HBsAg subtypes have been recognized: *adw*, *ayw*, *adr*, and *ayr*. The antibody specificity of the anti-Id–induced anti-HBs response by which HBsAg determinants were recognized was determined by RIA using microtiter plates coated with HBsAg subtypes *adw*, *ayw*, and *adr*. Each of the four anti-Id–induced anti-HBs bound equally well the three different HBsAg subtypes (Table VI), indicating that the reactivities of the anti-Id–induced anti-HBs were directed to the *a* determinant. This substantiates our previous observation (13) in that the binding of anti-Id antibodies was equally inhibited by preincubating the anti-HBs Id with three of the four HBsAg subtypes, indicating that the *a* determinant was responsible for the induction of the Id.

TABLE V

*Expression of the Interspecies Idiotype in Mouse Anti-HBs**

First injection	Second injection	Anti-HBs titer‡	Percent inhibition of the Id-anti-Id reaction
Anti-Id	HBsAg	6,250	52
		250	32
		6,250	61
		6,250	60
Pre-IgG	HBsAg	250	28
		>5	2
		>5	5
		25	19

* Mice received either 5 μg of alum-precipitated anti-Id or pre-IgG on day 0, followed by 6 μg of HBsAg on day 14. Mice were bled on day 26.

‡ Reciprocal dilution of antisera that gave a positive (S) to negative (N) cpm ratio of 2.1 (19).

TABLE VI
HBsAg Specificity of the Anti-Id–Induced Anti-HBs

First injection	Second injection	Reciprocal dilution of antisera tested	S/N ratio*		
			adw	*ayw*	*adr*
Anti-Id	Anti-Id	50	8.3	7.8	7.7
		1,250	1.8	1.5	1.6
Anti-Id	Anti-Id	50	8.0	7.8	7.8
		1,250	2.6	2.3	2.0
Anti-Id	Anti-Id	50	7.9	8.4	7.7
		1,250	3.6	3.3	4.4
Anti-Id	Anti-Id	50	7.9	7.9	8.2
		1,250	2.7	4.1	4.0
Pre-IgG‡	Pre-IgG	10	<1.8	<1.6	<1.8

* The calculation of the positive (S) to negative (N) ratio was done as previously described (19).
‡ These data represent a composite of four mice that each received two injections of pre-IgG.

The fact that the anti-Id–induced anti-HBs recognized the group-specific *a* determinant of HBsAg lends evidence that anti-Id antibodies can induce protective immunity against infectious HBV. In this light, it was demonstrated that antibodies directed against the *a* determinant confer protection in humans against HBV infection (32). No significant binding to the three HBsAg subtypes was obtained with sera from the four mice that received two injections of pre-IgG alone.

The theoretical implications for the use of anti-Id antibodies as vaccines for infectious agents have been previously proposed and discussed elsewhere (33–35). To our knowledge, the only experimental evidence that anti-Id can induce protective immunity against an infectious agent has been reported for African trypanosomiasis in mice (8). A second possibility of anti-Id–induced immunity appears with HBV. Previous studies (36) have demonstrated that anti-HBs is responsible for protection against challenge or reinfection with HBV. The fact that anti-HBs can be induced by the injection of anti-Id alone and that this anti-HBs expresses an idiotypic determinant shared by anti-HBs produced in humans naturally infected with HBV suggests the potential for the use of anti-Id vaccines for HBV infections. In addition, the anti-HBs also recognized the group-specific *a* determinant of HBsAg, which is the epitope that induces protective immunity against HBV. This possibility must await further testing, since only chimpanzees and humans can be infected with human HBV. Alternatively, anti-Id could be used in conjunction with HBsAg to potentiate the anti-HBs response to a single injection of HBsAg by priming the host's immune system before antigenic stimulation. These studies using mice to induce anti-HBs by injection of anti-Id represent an attempt to understand the humoral immune response to HBsAg via modulation of an Id network. It is not known whether the anti-HBs produced

in the modulation process described in this report resulted from the expression of clones secreting an Ab-3 (anti–anti-Id antibodies) which expresses the Id and binds antigen (33) or represents an internal image (33, 35) of HBsAg in which classic antigenic stimulation does not occur.

Summary

BALB/c mice receiving antiidiotype antibodies before the injection of hepatitis B surface antigen (HBsAg) generated an enhanced anti-HBs response. Mice given antiidiotype antibodies in a soluble form induced predominantly IgM anti-HBs, whereas alum-precipitated antiidiotype produced primarily IgG anti-HBs. Injection of antiidiotype antibodies alone induced anti-HBs that inhibited a common interspecies anti-HBs idiotype-antiidiotype reaction and recognized the group-specific determinant of HBsAg. These data support the view that antiidiotype antibodies may modulate the immune response to an infectious viral agent.

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