

## ENHANCED ANTIGEN IMMUNOGENICITY INDUCED BY BISPECIFIC ANTIBODIES

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T<sub>H</sub> cells recognize and respond to processed antigen bound to class II MHC molecules on the surfaces of APC (1, 2). In vitro, the targeting of antigen to APC surfaces by heterocrosslinked bispecific antibodies (HBAs)<sup>1</sup> greatly increases the efficiency with which APC endocytose, process, and present antigen to T cells (3-5). Since antibody responses against most protein antigens require T cell help in vivo, we have asked here if HBAs could also enhance the ability of an antigen, in this case hen egg lysozyme (HEL), to induce an antibody response in mice. HBAs were prepared by chemically crosslinking an antibody with specificity for HEL to various other antibodies, each specific for a particular APC cell surface component. Normally, the generation of immune responses after immunization with vaccines and other antigens requires relatively large amounts of antigen, multiple injections, and, in experimental animals, adjuvants (6, 7). By contrast, we show that HBAs, when administered once with nanogram amounts of antigen, in the absence of adjuvant, induce high titers of antibody in mice, and prime mice for a secondary IgG antibody response when rechallenged with soluble antigen.

### Materials and Methods

*Immunizations and Measurements of Antibody Production.* Mice of various strains were given primary injections containing various amounts of HEL (Sigma Chemical Co., St. Louis, MO) or HEL mixed with 5-10  $\mu$ g of HBA in PBS, in two portions of 50  $\mu$ l in each hind footpad. Controls received PBS alone. Mice were bled 21 d after the primary injection, and the next day given a secondary challenge of 5-10  $\mu$ g of HEL (5  $\mu$ g per hind footpad). 11 d later, the mice were bled again, sera were isolated, and anti-HEL IgG antibody was measured using a solid-phase ELISA. In the ELISA, HEL was bound to plastic microtiter wells and incubated sequentially with dilutions of each test serum, biotin anti-mouse IgG (Southern Biotechnology Assoc., Birmingham, AL), avidin-alkaline phosphatase (Sigma Chemical Co.), and finally, substrate (Sigma Chemical Co.). The amount of antibody in each serum was interpolated from the dose-response curve of a standard pool of hyperimmune sera, giving an arbitrary value of antibody U/ml.

*Antibodies.* All HBAs used in this paper were prepared by chemically crosslinking mAbs with succinimidyl-3-(2-pyridyldithiol)propionate as described (8). HBAs were separated from uncrosslinked monomeric antibodies by gel filtration and are designated "antibody 1  $\times$  anti-

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<sup>1</sup> Abbreviations used in this paper: HBA, heterocrosslinked bispecific antibodies; HEL, hen egg lysozyme.

body 2." For example, anti-I-A<sup>k</sup> × anti-HEL refers to a gel filtration fraction of 300 kD or greater, obtained when anti-I-A<sup>k</sup> was crosslinked to anti-HEL. Antibodies used in this paper are anti-HEL, HyHEL-8 (9); anti-I-A<sup>k</sup>, 10-2.16 (10); anti-I-A<sup>d</sup>, MKD6 (11); anti-K<sup>d</sup>D<sup>d</sup>, 34.1.2 (12); anti-K<sup>k</sup>, 36.7.5 (12); anti-Fc<sub>γ</sub>RII, 2.4G2 (13); anti-IgD<sup>a</sup>, AMδ28.1 (14); and anti-IgD<sup>b</sup>, AF3-33.3 (14). Fab fragments were produced by papain digestion and were purified by gel filtration followed by ion exchange chromatography (15).

### Results and Discussion

Female A/J mice were given subcutaneous injections of graded amounts of HEL or HEL mixed with HBAs, on day 0. 21 d later, the mice were bled to determine serum anti-HEL IgG antibody levels. On day 22, the mice were given booster injections of 5 μg of HEL per hind foot pad, to induce secondary IgG antibody responses. Sera were collected 11 d later and IgG antibody was measured. Fig. 1 shows that >10 μg of HEL per mouse was required in a primary injection to produce either a primary or a secondary IgG antibody response, if the HEL was given alone. However, in the presence of 10 μg of HBA (anti-I-A<sup>k</sup> × anti-HEL), 100 ng of HEL induced a substantial primary IgG antibody response, and as little as 10 ng of HEL induced a strong secondary response. In other experiments (e.g., Fig. 2), <1 ng of HEL in the presence of HBA gave a significant secondary response. Mice receiving PBS as a primary injection, and then given a secondary challenge of HEL (Fig. 1), or given the HBA alone as a primary injection and then a secondary challenge of HEL (Fig. 2), produced little or no IgG antibody. We compared immunization using HBAs with that of HEL emulsified in IFA (Fig. 2). The two types of immunization produced similar amounts of IgG antibody in the secondary response, especially at low HEL doses. Thus, the HBA specific for HEL and class II MHC structures increased the efficiency of immunization by 300-fold in the primary response, and by a factor of 10<sup>3</sup> to 10<sup>4</sup> after a secondary boost. The HBA increased the efficiency of priming for secondary IgG antibody responses at least as well as IFA.

We constructed several other HBAs that could bind to different surface molecules

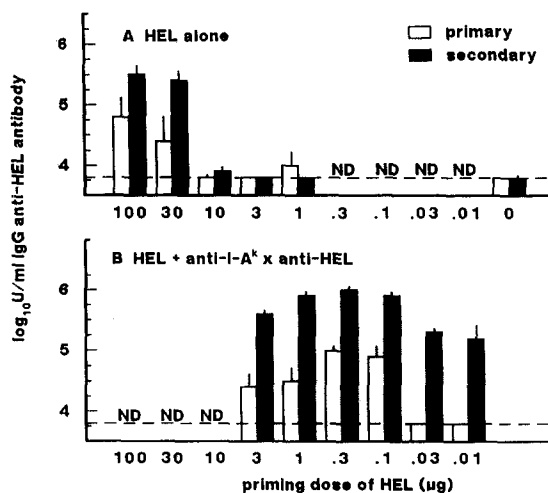


FIGURE 1. HBA enhancement of antibody responses in vivo. Groups of three female, 7-wk-old A/J mice were given single primary foot pad injections of (A) HEL alone or (B) HEL mixed with 10 μg HBA. The open bars indicate serum IgG anti-HEL antibody levels 21 d after the primary injection. The solid bars indicate antibody levels in sera taken 11 d after these same mice were given a secondary injection of 10 μg HEL without the HBA. The dashed line indicates the lower limit of detection.

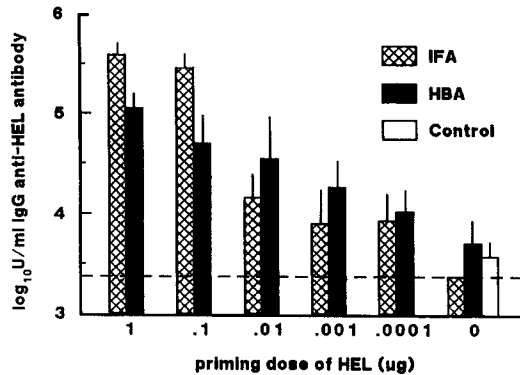


FIGURE 2. Enhancement of antibody production by HBA and IFA. Secondary antibody responses are shown for groups of four female, 6-wk-old B10.A mice given primary injections of HEL mixed with 10 µg HBA (solid bars) or emulsified (1:1 [vol/vol]) in IFA (cross-hatched bars). Control mice (open bar) received HBA but no HEL as a primary injection. The horizontal dashed line indicates the limit of detection of antibody.

on various APC, and used them to immunize mice (Table I). HBAs specific for class I or class II MHC molecules, or Fc<sub>γ</sub>RII, all enhanced the ability of HEL to induce antibody responses in different strains of mice. However, two HBAs with specificity for two allotypes of IgD when tested in several strains of mice induced little or no anti-HEL antibody (Table I, Exp. 2). In other experiments (not shown), we were unable to generate a significant anti-HEL response by varying the dosages of the IgD-specific HBAs and HEL from 20 to 0.1 µg. These same IgD-specific HBAs enhance the efficiency of presentation of HEL to Th hybridomas by B cells in vitro

TABLE I  
HBA with Specificity for Class I or II MHC Molecules and for Fc<sub>γ</sub>RII,  
but not for sIgD, Enhance Humoral Responses Against HEL

Exp.	Mouse strain	Haplotype KID	HBA in primary injection	IgG anti-HEL responses	
				Primary	Secondary
<i>U/ml × 10<sup>-3</sup></i>					
1	CAF1	ddd × kkd	-	7(0)*	7(0)
			Anti-I-A <sup>k</sup> × anti-HEL	8(1)	166(60)
			Anti-K <sup>d</sup> D <sup>d</sup> × anti-HEL	7(0)	33(11)
	A/J	kkd	Anti-Fc <sub>γ</sub> RII × anti-HEL	8(1)	40(15)
			-	ND	9(4)
			Anti-I-A <sup>k</sup> × anti-HEL	63(15)	933(200)
		Anti-K <sup>k</sup> × anti-HEL	14(12)	302(115)	
		Anti-Fc <sub>γ</sub> RII × anti-HEL	13(11)	269(70)	
2	CAF1	ddd × kkd	-	2(0)	3(2)
			Anti-I-A <sup>k</sup> × anti-HEL	19(6)	118(14)
			Anti-IgD <sup>a</sup> × anti-HEL	2(0)	9(4)
	B10.BR	kkk	-	2(0)	3(0)
			Anti-I-A <sup>k</sup> × anti-HEL	19(10)	39(5)
			Anti-IgD <sup>b</sup> × anti-HEL	3(1)	12(2)

Groups of 4-5 female mice of different strains were given primary injections of 2 µg of HEL with or without 10 µg of HBA, having the indicated specificities. After 21 d, each mouse was given a second injection of 10 µg of HEL in PBS. Sera were collected 11-13 d later, and anti-HEL IgG antibody was measured and is shown as U/ml. The limits of detection were 7,000 U/ml for Exp. 1 and 2,000 U/ml for Exp. 2.

\* Figures in parentheses are SEM.

(4). The inability of anti-sIgD-containing HBAs to enhance immunogenicity suggests that either these HBA antigen complexes, when given subcutaneously, do not bind to B cells *in vivo* (e.g., they might be cleared rapidly or circulating IgD might block their binding to B cells), or that B cells are not able to stimulate unprimed T cells *in vivo*. B cells appear to serve as *in vivo* APC to stimulate T cell proliferation in other systems (16–20).

Several important controls for the effect of HBAs are shown in Table II. First, HBAs enhanced antibody production only if the appropriate antibodies were in fact crosslinked; monomeric mixtures of antibodies did not work (Table II, Exp. 1). Second, antibody was induced only if the strain of mouse immunized expressed the structures to which the HBA could bind (Table II, Exp. 2). Thus, HBAs with I-A<sup>d</sup> or K<sup>d</sup>D<sup>d</sup> specificities did not produce responses in H-2<sup>k</sup> (C3H) mice, but did produce responses in H-2<sup>k</sup> × H-2<sup>d</sup> (CAF1) mice. The same I-A<sup>d</sup>-specific HBA did not induce antibody in A/J mice, but the K<sup>d</sup>D<sup>d</sup>-specific HBA did, since this strain expresses D<sup>d</sup> but not I-A<sup>d</sup>. Both the C3H and the A/J mice responded when immunized with the HEL- and Fc<sub>γ</sub>RII-specific HBA.

Because antigen targeted to Fc<sub>γ</sub>RII gave enhanced antibody responses to HEL in several strains, it was important to show that the HBAs were not simply forming immune complexes with HEL and binding via their Fc portions to Fc<sub>γ</sub>R on APC. Therefore, we constructed an HBA from Fab fragments of anti-HEL and anti-I-A<sup>k</sup> mAbs, and compared its ability to induce anti-HEL antibody with that of its intact Ig counterpart (Table II, Exp. 3). The results show that the Fab-HBA performed as well as the intact Ig-HBA over a range of dosages, indicating that the Fc regions of the HBA were not required for enhanced antibody production. This conclusion is also supported by the observation that anti-H-2<sup>d</sup>-specific HBAs did not promote antibody production in H-2<sup>k</sup> (i.e., C3H) mice (Table II, Exp. 2).

It is likely that HBAs increase anti-HEL production in mice by targeting HEL to surface structures on APC *in vivo*. Such targeting would cause the antigen to be processed and presented to T cells with much higher efficiency than antigen given alone, as documented *in vitro* (3–5). Presumably, such APC could then generate large numbers of antigen-specific Th cells, which in turn would stimulate antigen-specific B cells. The known T cell dependence of anti-HEL responses in mice (21, 22) supports this view.

Our use of HBAs to enhance immunization follows on previous results (18, 23–25), showing that antigens that were chemically crosslinked directly to antibodies specific for class II MHC or IgM molecules were more potent immunogens than antigens given alone. HBAs provide several important advantages over chemically linked antigen-antibody conjugates. First, HBAs could be used to enhance responses against small amounts of impure antigens, for example, tumor antigens, whereas direct linkage of antigen to antibody requires relatively large amounts of highly purified antigen. HBAs do not require biochemical modification of antigen, which could alter its immunogenicity. In addition, bispecific antibodies in the form of hybrid-hybridomas (26, 27) or hetero-F(ab)<sub>2</sub> (28) can be prepared as single homogeneous species, whereas antibody-antigen chemical complexes are heterogeneous, and vary between preparations. Therefore, homogeneous bispecific antibodies would be much more suitable for large scale production than the antigen-antibody chemical complexes. Finally, we have found that the microgram amounts of HBAs used in this study are

TABLE II  
Form, Specificity, and Dose of HBA Required for Enhancement of Humoral Responses

Exp.	Mouse strain	Antibody (form)	Ab dose	HEL dose	Secondary response: IgG anti-HEL	
			$\mu\text{g}$	$\mu\text{g}$	$\text{U/ml} \times 10^{-3}$	
1	A/J	Anti-I-A <sup>k</sup> × anti-HEL (HBA)	5	1	250(59)*	
		Anti-I-A <sup>k</sup> + anti-HEL (mix)	5	1	9(3)	
		Anti-Fc <sub>γ</sub> RII × anti-HEL (HBA)	5	1	120(21)	
		Anti-Fc <sub>γ</sub> RII × anti-HEL (mix)	5	1	11(2)	
		Anti-HEL (mAb)	2.5	1	10(3)	
		None	0	1	11(6)	
		None	0	0	7(0)	
2	CAF1	Anti-I-A <sup>d</sup> × anti-HEL (HBA)	10	3	240(65)	
		Anti-K <sup>d</sup> D <sup>d</sup> × anti-HEL (HBA)	10	3	174(70)	
		None	0	3	3(0)	
	C3H/HeJ	Anti-I-A <sup>d</sup> × anti-HEL (HBA)	10	3	8(4)	
		Anti-K <sup>d</sup> D <sup>d</sup> × anti-HEL (HBA)	10	3	5(3)	
		Anti-Fc <sub>γ</sub> RII × anti-HEL (HBA)	10	3	245(95)	
		None	0	3	11(5)	
	A/J	Anti-I-A <sup>d</sup> × anti-HEL (HBA)	10	3	18(9)	
		Anti-K <sup>d</sup> D <sup>d</sup> × anti-HEL (HBA)	10	3	112(69)	
		Anti-Fc <sub>γ</sub> RII × anti-HEL (HBA)	10	3	302(122)	
		None	-	3	13(2)	
	3	A/J	Anti-I-A <sup>k</sup> × anti-HEL (HBA)	1	2	417(129)
			Anti-I-A <sup>k</sup> × anti-HEL (HBA)	3	2	525(183)
Anti-I-A <sup>k</sup> × anti-HEL (HBA)			10	2	372(55)	
Anti-I-A <sup>k</sup> × anti-HEL (Fab-HBA)			1	2	1,122(440)	
Anti-I-A <sup>k</sup> × anti-HEL (Fab-HBA)			3	2	603(209)	
Anti-I-A <sup>k</sup> × anti-HEL (Fab-HBA)			10	2	587(124)	
None			0	2	1(0)	

Groups of female mice (4-5 per group) were injected with the indicated doses of HEL or antibody. In Exp. 1, the effect of HBAs on the anti-HEL antibody response was compared with that of the uncross-linked fraction (mix) obtained during purification of HBAs, and to that of the anti-HEL mAb alone. In Exp. 2, the specificity of targeting was tested in mouse strains that express structures to which particular HBAs bind or do not bind. C3H/HeJ and A/J mice were also immunized with HEL plus an anti-Fc<sub>γ</sub>RII-specific HBA as a control for their ability to respond to targeted HEL. The isotypes and allotypes of antibodies used in this experiment were as follows: anti-I-A<sup>d</sup> (MKD6), mouse IgG2a, IgH1-b or -e (strain of origin, B6 × A/J); anti-K<sup>d</sup>D<sup>d</sup> (34.1.2), mouse IgG2a, IgH1-j (strain of origin, C3H); anti-HEL (HyHEL-8), mouse IgG1, IgH4-a (strain of origin, BALB/C); anti-Fc<sub>γ</sub>RII (2.4G2), rat IgG2b. There is no obvious relationship between possible alloreactivities against HBAs and their ability to enhance anti-HEL production. In Exp. 3, anti-I-A<sup>k</sup> × anti-HEL HBAs prepared from intact antibodies or from Fab fragments were compared for their ability to induce anti-HEL antibody. In all experiments, mice were bled 11-14 d after a secondary injection of 10  $\mu\text{g}$  of HEL in PBS. Data are mean U/ml of serum IgG antibody. The limits of detection were 7,000 U/ml (Exp. 1), 3,000 U/ml (Exp. 2), and 1,000 U/ml (Exp. 3).

\* Figures in parentheses are SEM.

remarkably potent enhancers of immunogenicity, leading to priming with nanogram amounts of antigen. Such enhancements of immunogenicity are far greater than those reported in studies using antigen-antibody chemical complexes. Our observations that relatively small amounts of HBAs can enhance antibody responses at least as well as IFA suggests that HBAs may be useful in immunizing man and domestic animals against bacterial, viral, parasitic, and, perhaps, tumor antigens. Such im-

munization would be efficient and would avoid the use of adjuvants that have unwanted inflammatory effects (29), and cannot, therefore, be used in humans.

### Summary

The binding of protein antigens to APC with heterocrosslinked bispecific antibodies (HBAs) enhances their processing and presentation to Th cells in vitro. Here we have asked whether HBAs could also increase immune responses in vivo. We immunized mice with hen egg lysozyme (HEL) in the presence or absence of HBA, and followed antibody production after the primary challenge and after a secondary boost. We found that HBAs that bind antigen to MHC class I or II molecules, to Fc $\gamma$ R, but not to surface IgD, enhance the immunogenicity of HEL. HBAs that bound HEL to MHC class II molecules, for examples, decreased the amount of antigen required to elicit a primary anti-HEL antibody response in mice by 300-fold, and the amount required to prime for a secondary response by 10<sup>3</sup>- to 10<sup>4</sup>-fold. In fact, HBAs were as effective as IFA in generating antibody responses. Since adjuvants cannot be used in humans, HBAs could prove useful for immunizing people, especially in cases where, due to scarcity or toxicity, minute doses of antigen must be used.

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