

BYSTANDER HELP IN PRIMARY IMMUNE RESPONSES IN VIVO

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Two distinct pathways for T cell-dependent B cell activation have been described (1, 2) in a variety of experimental systems that measure secondary antibody responses. Cognate help is characterized by a requirement for covalent linkage of determinant(s) recognized by Th cells and determinant(s) recognized by the responding B cells (3-6). The carrier effect illustrates this pathway, in which secondary, hapten-specific antibody responses require that hapten be coupled to the same carrier used for initial priming (7-10). On the other hand, in bystander help, Th cells primed to a particular carrier in the presence of that carrier support secondary antibody responses to hapten linked to a different, noncrossreactive antigen (6, 11-22). Bystander help has generally been difficult to show *in vivo* or for protein-bound indicator antigens (21-24). Thus, it has been proposed that this mechanism of T cell-B cell collaboration may be of little importance *in vivo* (25, 26).

The murine immune response to heterologous insulins is controlled by H-2-linked immune response (I_r) genes (27-31). Responsiveness is ultimately determined by the interaction of carrier-specific regulatory T cells (32-36). In the present communication, we investigate a puzzling phenomenon Keck (29) saw during coimmunization experiments using insulins. Keck reported that H-2^k mice, which respond to sheep but not pork insulin, developed hapten-specific antibody after immunization with a mixture containing haptened pork insulin and sheep insulin. These animals did not respond to haptened pork insulin alone. Thus, primary hapten-specific antibody responses did not require covalent linkage of the hapten to the immunogenic carrier, sheep insulin. We find that this phenomenon is reproducible and that, in fact, it represents an *in vivo* example of induction of primary, T cell-dependent antibody responses through a bystander mechanism of help.

Materials and Methods

Mice. C57BL/10 (B10), C57BL/6 (B6), B10.A, B10.Q, BALB.B, and CBA/N mice were bred and maintained at the Animal Resources Facility of the Jewish Hospital of St. Louis, St. Louis, MO. These mice were vaccinated with IHD-T strain of vaccinia virus at 5 wk of age (37). All mice were maintained in accordance with National Institutes of Health guidelines.

Antigens and Immunization. Pork and beef insulins were purchased from Elanco

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Products Co., Indianapolis, IN. Sheep insulin and mouse hemoglobin were purchased from Sigma Chemical Co., St. Louis, MO. Fowl gamma globulin (FGG)¹ was purified from chicken serum obtained from the Colorado Serum Co., Denver, CO. Pigeon cytochrome *c* was the generous gift of Dr. Ron Schwartz, National Institute of Allergy and Infectious Diseases, Bethesda, MD. Fluorescein (Fl) was coupled to insulin, FGG, mouse hemoglobin, or pigeon cytochrome *c* as described (38). Fl-FGG contained 5.5 molecules Fl per molecule of FGG. Other Fl antigen preparations contained 0.5–1.0 molecules of Fl per molecule of antigen as shown by spectrophotometric analysis. Mice were immunized with 50 μ g antigen, or mixture containing 50 μ g of each antigen, in a 1:1 vol/vol emulsion of IFA or CFA (Difco Laboratories Inc., Detroit, MI). T cell donors for cell transfer experiments were injected with 50 μ g antigen in CFA in the hind footpads 10–20 d before cell transfer. B cell donors received an initial 50 μ g i.p. injection of antigen in CFA and were boosted intraperitoneally with 20 μ g soluble antigen. Spleen cells were harvested no less than 8 wk after initial immunization. In some experiments, antigen-specific unresponsiveness was induced by 100 μ g i.v. injection of beef insulin 7 d before challenge with antigen in CFA (39).

Preparation of Lymphocyte Populations and Cell Transfer. Splenic and lymph node cells were separated into B T cell fractions as described (38). Briefly, B cell donors were injected with 0.4 ml of a 1:10 dilution of antithymocyte serum 2 d before they were killed. Splenic B cells were enriched by incubation with monoclonal rat anti-Thy-1 (T24/40.7 obtained from the Salk institute) and prescreened rabbit serum as a source of complement (Cedarlane Laboratories, Westbury, NY). T cells were enriched from lymph node lymphocytes by adsorption of B cells onto goat anti-Ig-coated petri dishes using a modification of the technique of Mage et al. (40). T cells were collected as nonadherent cells. Cells were injected intravenously into syngeneic, sex- and age-matched mice (33). For some experiments, recipient mice were irradiated with 650 rad delivered by a ¹³⁷Cs source (Gamma-cell-40; Atomic Energy of Canada, Ltd., Ottawa, Canada) before cell transfer. Mice were challenged intraperitoneally with antigen in CFA 24 h after transfer.

ELISA. Serum antibodies were determined using a modification of the ELISA described by Engvall and Perlmann (41). Polyvinyl microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with 10 μ g/ml beef insulin, Fl-gelatin, or mouse hemoglobin in borate buffered saline (pH 9.0) for 18 h at 4°C, and excess free-protein binding sites on the plastic wells were saturated with PBS containing 1 mg/ml BSA for 1–2 h. Plates were washed with saline and incubated overnight with sera that had been serially diluted in PBS containing 1 mg/ml BSA. The plates were washed and incubated for 1½ h at 37°C with alkaline phosphatase coupled to goat anti-mouse Ig antibody. The plates were washed again and incubated at room temperature with 0.6 mg/ml *p*-nitrophenyl phosphate in buffer containing 1.0 M diethanolamine and 0.2 mM MgCl₂. The OD at 405 nm was read on a Microelisa Auto Reader (Dynatech Laboratories, Inc.). Titers were calculated from the serum dilution at which OD was 0.1. Controls included normal mouse serum, which gave negligible readings, and serum from immunized mice as a positive control to standardize the assay. Results are expressed on the arithmetic means \pm SEM of antibody titers of three or more mice per group.

Hemolytic Plaque Assay. Fl was coupled to SRBC by the method of Caldron et al. (42) using FITC. IgM PFC were inhibited with goat anti-mouse μ chain serum (Gateway Immunosera, Cahokia, IL). IgG PFC were facilitated with subclass-specific antisera (Litton Bionetics, Inc., Kensington, MD). The isotype specificity of these antisera was confirmed with myelomas. Fl-specific PFC were determined by enumerating PFC on FL-SRBC and subtracting the number of PFC detected in the presence of 25 μ g/ml Fl-coupled to E-amino-n-caproic acid (38).

Results

Bystander Help in the Primary Antibody Response to Hapten-coupled Insulin. Mice of the *H-2^b* or *H-2^k* haplotypes are nonresponders to pork insulin and responders

¹ Abbreviations used in this paper: FGG, fowl gamma globulin; Fl, fluorescein.

Species	DIFFERENCES IN AMINO ACID SEQUENCES OF INSULIN								ANTIBODY RESPONSES			
	B Chain			A Chain					by			
	B4	B10	B31	A4	A8	A9	A10	H-2 ^d	H-2 ^b	H-2 ^k	H-2 ^a	
Mouse I	-Lys	Pro*	Ser	-Asp	Thr	Ser	Ile	NT	NT	NT	NT	
Rat I								-	-	-	NT	
Pork	-Asn	Ser	Ala	-Glu				+	-	-	-	
Beef	-Asn	Ser	Ala	-Glu	Ala		Val	+	+	-	-	
Sheep	-Asn	Ser	Ala	-Glu	Ala	Gly	Val	+	+	+	-	
Horse	-Asn	Ser	Ala	-Glu			Gly	+	+	+	-	

FIGURE 1. Amino acid sequences and immunogenicity of insulin. Insulin sequences were derived from Dayhoff (48), and responsiveness to different insulin species was determined by Keck (27-29), Kapp and Strayer (32), and Bucy and Kapp (33). Asterisk indicates that mouse and rat insulin II have Ser at residue B10. NT, not tested.

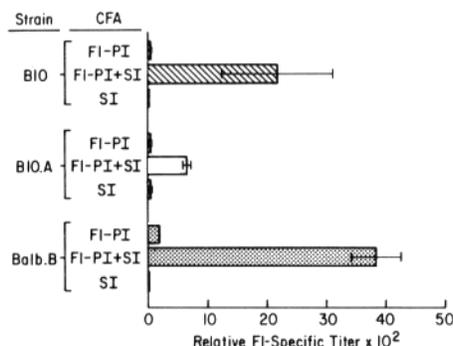


FIGURE 2. Coimmunization overrides genetic control of antibody responses to FI-pork insulin. Mice were injected in the hind footpads with 50 μ g FI-pork insulin (FI-PI), sheep insulin (SI), or a mixture containing 50 μ g of each in emulsion with CFA. Serum antibody titers were measured 14 d later using a FI-specific ELISA.

to sheep insulin (Fig. 1). Haptenated pork insulin is, therefore, poorly immunogenic in these animals; little if any hapten-specific antibody is produced after immunization with FI-pork insulin (Fig. 2). However, immunization with a mixture of FI-pork insulin and immunogenic sheep insulin induces a significant titer of FI-specific antibody in B10, B10.A, and BALB.B mice (Fig. 2). Both antigens must be injected in the same anatomical location, but there is no requirement for mixing of the antigens before immunization. Separate intraperitoneal injections of each antigen will induce hapten-specific antibody responses (unpublished data). Thus, it is unlikely that the data can be explained by a physical transfer of fluorescein from one protein to another. These results confirm those of Keck (29), and suggest that hapten-carrier linkage is not required for the induction of primary antibody responses under the experimental conditions used.

This phenomenon was initially interpreted to result from crossreactivity between pork and sheep insulins. If this interpretation were correct, mixtures containing FI-pork insulin and proteins, other than insulin variants would not induce FI-specific antibody; we observed the contrary. Mice of the H-2^b haplotype produced FI-specific antibody after immunization with mixtures of FI-pork insulin and FGG (Fig. 3). We also saw this result when using other proteins in place of FGG and other low responder strains of mice (unpublished data). Note

BYSTANDER HELP IN PRIMARY RESPONSES

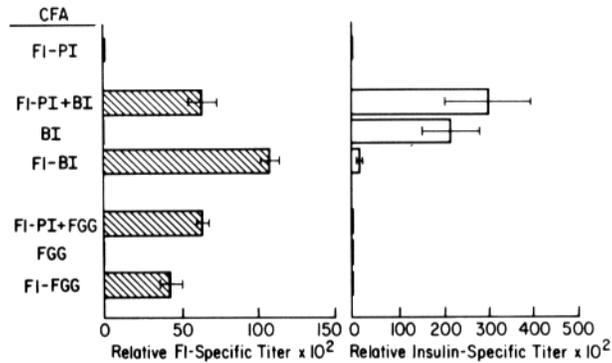


FIGURE 3. Bystander help can be induced by FGG. B10 mice were immunized in the hind footpads with 50 μ g FI-pork insulin (FI-PI), beef insulin (BI), FGG, FI-FGG, or mixtures containing 50 μ g of each antigen; they were bled 14 d later for determination of serum antibody.

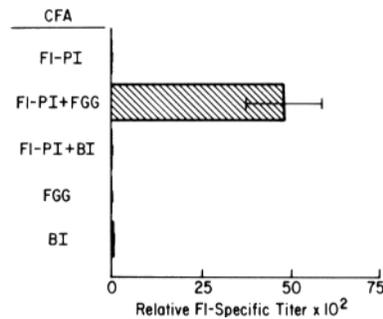


FIGURE 4. The coimmunogen must be immunogenic. The same procedure was followed as described in the legend to Fig. 3, except that B10.Q mice were used. Beef insulin is not immunogenic in this strain.

that beef insulin, which is immunogenic in $H-2^b$ mice, could induce bystander help (Fig. 3). FI-specific bystander responses thus were readily shown after immunization with a variety of antigenic mixtures. In contrast, mixtures of FI-pork insulin plus FGG did not induce measurable insulin-specific antibody in $H-2^b$ mice (Fig. 3). We further address this observation in the next section.

Subsequent experiments showed that the "coimmunogen" must be immunogenic. Neither beef nor pork insulin are immunogenic in $H-2^g$ mice (Fig. 1), and these mice develop FI-specific antibody after coimmunization with FI-pork insulin mixed with FGG, but not with mixtures containing beef insulin (Fig. 4). This result contrasted with that obtained using $H-2^b$ mice, which are responders to beef insulin (Fig. 3). Thus, an antigen (beef insulin) that was perceived as nonimmunogenic by virtue of Ir gene control, failed to induce bystander help. We therefore tested whether a normally immunogenic antigen could induce bystander help in mice that were tolerant to that antigen. Antigen-specific T cell tolerance can be induced by intravenous injection of insulin (39). Tolerance induced in this manner is associated with reversible T cell anergy, rather than induction of suppressor cells. Pretreatment of $H-2^b$ mice with an intravenous injection of beef insulin inhibits the insulin-specific antibody response to beef

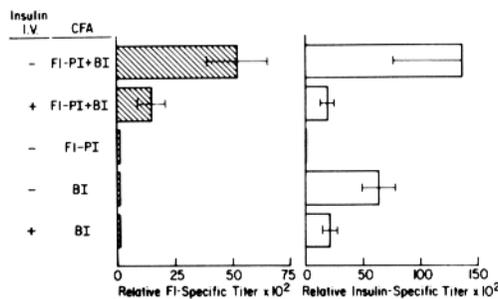


FIGURE 5. T cell tolerance reduces the ability of the coimmunogen to support bystander help. B10 mice were rendered tolerant to beef insulin by injection of 100 μg of beef insulin i.v. (39). After 7 d, tolerant mice and control mice, which had been injected with saline, were challenged as described in the legend for Fig. 3. Serum was evaluated for FI-specific and insulin-specific antibody 14 d after challenge.

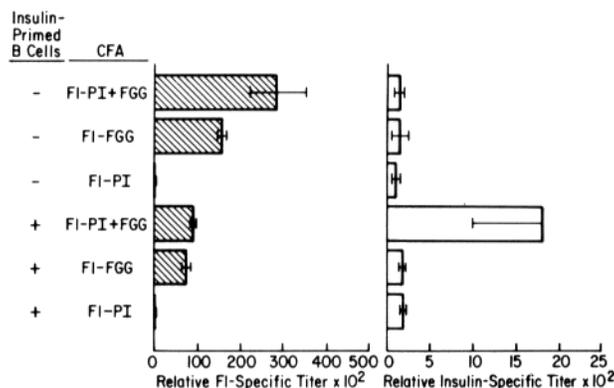


FIGURE 6. Induction of insulin-specific antibody by coimmunization. Unirradiated B10 mice were inoculated intravenously with 2×10^7 syngeneic B cells (T cell-depleted splenocytes) from donors primed and boosted with beef insulin. 4 d after cell transfer recipients and control animals were challenged intraperitoneally with 50 μg FI-pork insulin (FI-PI), FI-FGG, or with mixtures containing FI-pork insulin and FGG in emulsion with CFA. Serum was evaluated for antibody 21 d after challenge.

insulin in CFA (Fig. 5). This tolerization protocol also inhibits the FI-specific antibody response induced by mixtures of FI-pork and beef insulin. Thus, tolerance to the coimmunogen inhibits the ability to support bystander help.

B Cell Subsets Induced by Coimmunization. We were initially puzzled by the observation that while significant titers of FI-specific antibody were induced after coimmunization with FI-pork insulin and FGG, little if any insulin-specific antibody was detected (Fig. 3). This raised the possibility that the regulation of antibody responses to determinants intrinsic to the insulin molecule might differ from regulation of FI-specific antibody responses. Alternatively, the absence of insulin binding antibody after coimmunization might be attributed to a relative paucity of insulin-specific B cell precursors; the data in Fig. 6 support this theory. Animals inoculated with syngeneic B cells from insulin-primed cell donors produced insulin-binding antibody after immunization with a mixture of FI-pork insulin and FGG. A similar result was obtained in animals preimmunized with beef insulin and challenged with a mixture of pork insulin and FGG (Fig. 7). In

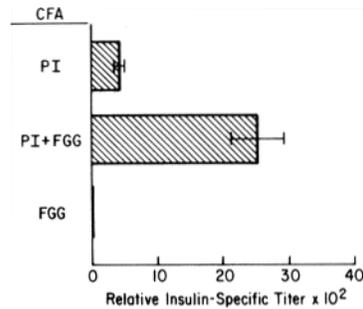


FIGURE 7. Induction of insulin-specific antibody by coimmunization. B10 mice were primed intraperitoneally with 50 μ g beef insulin in CFA and boosted intraperitoneally with 20 μ g soluble beef insulin 4 wk later. 12 wk after initial immunization the animals were challenged in the hind foot pads with 50 μ g pork insulin (PI), FGG, or pork insulin plus FGG in CFA. Insulin-specific serum antibody was determined 14 d after final challenge.

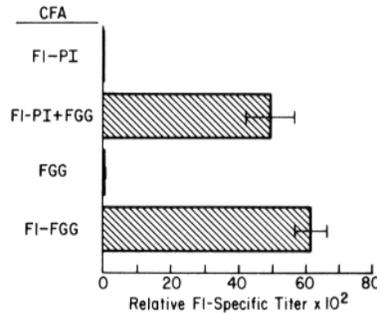


FIGURE 8. Bystander help in CBA/N mice. The same procedure was followed as described in the legend to Fig. 3, except that CBA/N mice were used.

both cases, B cells were expanded using an immunogenic variant of insulin that is known to be crossreactive with pork insulin at the B cell level (30). Thus, the magnitude of the bystander response induced by coimmunization appears to reflect the number of antigen-binding B cells available for activation.

Studies using CBA/N defective mice have shown two different pathways for in vitro T cell-dependent B cell activation involving distinct subsets of B cells (1, 2). Lyb-5⁻ B cells are activated by a mechanism that requires hapten-carrier linkage; in contrast, Lyb-5⁺ B cells, which are absent in CBA/N mice, do not require hapten-carrier linkage for in vitro, T cell-dependent activation. We further asked whether Lyb-5⁺ B cells were required for bystander help induced by coimmunization. CBA/N mice, which are low responders to pork insulin, produced FI-specific antibody after immunization with a mixture of FI-pork insulin and FGG (Fig. 8). The antibody titers stimulated by coimmunization were comparable to those induced by FI-FGG, indicating that Lyb-5⁺ B cells are not required for bystander help in the primary in vivo antibody response. Additionally, the isotype distribution of FI-specific antibody secreting cells induced by coimmunization paralleled that induced by FI-FGG (Fig. 9). In each case, IgG antibody predominated. We have thus been unable to distinguish bystander and cognate help on the basis of differential activation of distinct B cell subsets.

Kinetics of the Bystander Response. We (33) and others (3-6) have reported

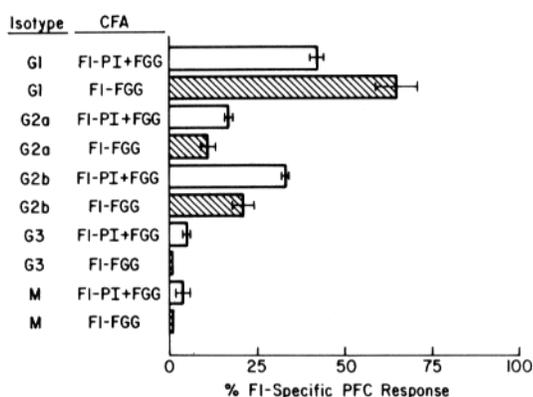


FIGURE 9. Isotype distribution of bystander vs. cognate help. BALB.B mice were immunized in the hind foot pads with 50 μ g FI-FGG or a mixture containing 50 μ g FI-pork insulin and 50 μ g FGG (FI-PI + FGG). FI-specific PFC were enumerated 14 d after challenge from the draining lymph node lymphocytes of individual animals, as described in Materials and Methods.

results of experiments measuring secondary antibody responses that conflict with those reported here. Adoptive recipients, reconstituted with beef insulin-primed T cells and FI-primed B cells, developed FI-specific antibody after challenge with FI-beef insulin, but not after challenge with a mixture of FI-pork insulin and beef insulin (33). This experiment was performed with *H-2^b* mice, which respond to beef but not pork insulin (Fig. 1). PFC responses were measured at a time point 9 d after challenge, which is optimal for secondary PFC responses in this system. In the same haplotype, coimmunization with FI-pork and beef insulin stimulates a primary hapten-specific antibody response (Fig. 3). The requirement for hapten-carrier linkage in secondary responses, as illustrated by the adoptive transfer experiment described above, has been confirmed in vitro (38 and unpublished data).

Together, the data suggested that hapten-carrier linkage was required in the secondary but not the primary antibody response. Primary antibody responses can be measured in adoptive recipients, but significant serum antibody titers do not arise before 20 d after challenge. To rule out the possibility that the requirement for hapten-carrier linkage in secondary responses is an artifact associated with cell transfer experiments, coimmunization experiments were performed with adoptive recipients. Animals reconstituted with CFA-primed T cells and FI-primed B cells were challenged with various antigens (Fig. 10). As expected, in recipients lacking carrier-primed T cells antibody was not measurable 9 d after challenge with FI-beef insulin. However, a primary antibody response was detected 23 d after challenge. Coimmunization of these recipients with nonimmunogenic FI-pork insulin and immunogenic beef insulin also induced a FI-specific antibody response with primary kinetics. Thus, cell transfer does not necessarily interfere with the ability to generate bystander help.

In comparison, recipients reconstituted with beef insulin-primed T cells and FI-primed B cells developed accelerated and augmented (secondary) antibody responses after challenge with FI-beef insulin such that high titers of FI-specific antibody were measurable 9 d after challenge (Fig. 10). Similarly reconstituted

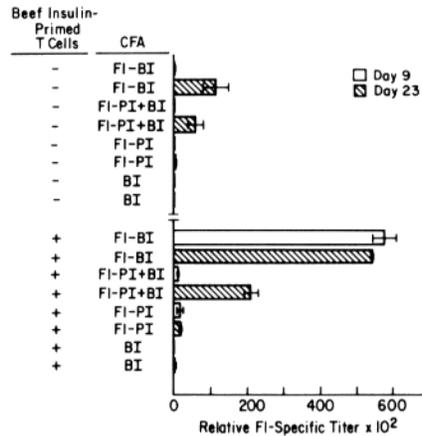


FIGURE 10. The kinetics of the bystander response. Irradiated (650 rad) B6 mice were reconstituted with 3×10^7 FI-FGG-primed splenic B cells and 2×10^7 lymph node T cells from donors that had been injected with saline/CFA or beef insulin/CFA. Recipients were challenged intraperitoneally the next day with $50 \mu\text{g}$ FI-pork insulin (FI-PI), beef insulin (BI), FI-beef insulin (FI-BI), or a mixture containing $50 \mu\text{g}$ FI-pork insulin and $50 \mu\text{g}$ beef insulin. FI-specific serum antibody was determined 9 and 23 d after challenge.

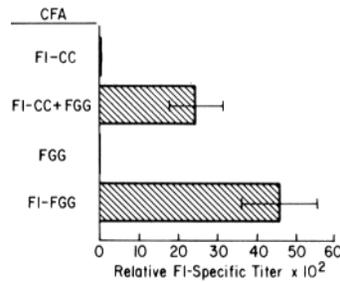


FIGURE 11. Coimmunization overrides genetic control of antibody responses to FI-pigeon cytochrome *c* (FI-CC). BALB.B mice were immunized with $50 \mu\text{g}$ antigen in CFA in the hind footpads, and FI-specific antibody was measured 14 d later.

recipients, challenged with a mixture of FI-pork insulin and beef insulin, had delayed antibody responses, developing significant titers 23 d after challenge. The response was augmented compared with that observed in recipients lacking beef insulin-primed T cells, but nevertheless retained the kinetic characteristics of primary antibody responses in this system. Thus, bystander help can be induced in "primed animals", but the kinetics of the response are not accelerated.

The General Significance of Coimmunization. We have shown that coimmunization can override the genetic control of antibody responses to pork insulin. Experiments were performed with other antigens to test whether H-2-linked Ir gene control also could be bypassed after coimmunization. Low responder BALB.B mice developed significant FI-specific antibody titers after immunization with a mixture of FI-pigeon cytochrome *c* and FGG, but not with FI-pigeon cytochrome *c* alone (Fig. 11). Similar results were obtained in low responders with FI-GAT and FI-lactate dehydrogenase (unpublished data). Additionally, genetic control of antibody responses to the hapten-modified self antigen, FI-

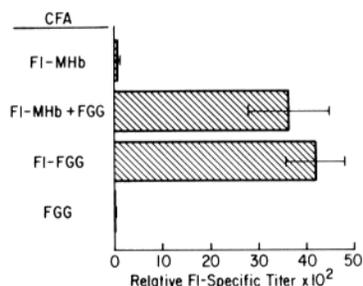


FIGURE 12. Coimmunization stimulates a response to FI-mouse hemoglobin (FI-MHb). Immunization and assay procedures were followed for B10 mice as described in the legend to Fig. 3.

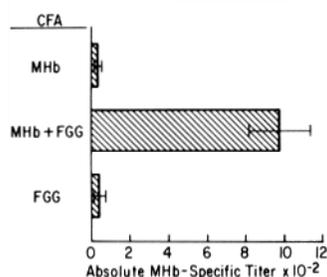


FIGURE 13. Autoantibody production after coimmunization. B10 mice were immunized with 50 μ g mouse hemoglobin (MHb), FGG, or a mixture containing 50 μ g of each in the hind footpads. MHb-binding antibody was determined 14 d after challenge.

mouse hemoglobin, is bypassed with coimmunization (Fig. 12). Immunization with a mixture of unmodified mouse hemoglobin and FGG stimulated mouse hemoglobin-binding autoantibodies (Fig. 13). These results suggested that bystander help can override both H-2-linked unresponsiveness and self tolerance.

Discussion

T cell-dependent B cell activation can proceed by two distinct pathways which differ in the requirement for covalent linkage of the determinants recognized by T cells and B cells (1, 2). These pathways have been shown in experimental systems that were dependent upon the measurement of secondary antibody responses. Motivated by the observations of Keck (27), we have used an experimental approach that allows one to evaluate the requirement for hapten-carrier linkage during primary antibody responses in vivo. This was accomplished by immunization with mixtures containing both nonimmunogenic and immunogenic proteins. The data indicate that Th cells can activate hapten-specific B cells during a primary immune response via a bystander mechanism that does not require hapten-carrier linkage. We have identified two factors that are critical for the induction of bystander help in the primary antibody response. The magnitude of the bystander response reflects the immunogenicity of the coimmunogen. Antigens with reduced immunogenicity by virtue of Ir gene control or tolerance induction are less able to induce bystander help after coimmunization. Induction of insulin-binding antibody in nonresponder animals immunized

with a mixture of pork insulin and FGG required prior manipulation to expand the insulin-specific B cell repertoire. Thus, the magnitude of the response is a function of the number of specific B cells available for activation.

Previous reports (1, 2) have suggested that distinct B cell subpopulations are preferentially activated by bystander help vs. cognate help. By measuring secondary *in vitro* antibody responses, others have shown that Lyb-5⁺ B cells are selectively activated in conditions favoring bystander help. CBA/N B cells, which are deficient in this subpopulation, required linked recognition for the induction of antibody secretion. In contrast, our results indicate that bystander help can be readily shown in the primary antibody response of CBA/N mice. Furthermore, no distinction could be made between primary bystander and cognate responses on the basis of the isotype distributions of these responses. We cannot, however, eliminate the possibility that subpopulations of B cells, differing in phenotype or state of activation, may be preferentially induced by coimmunization (18, 43).

Data from a number of groups have suggested that distinct, primed, Th cell subpopulations participate in cognate, vs. unlinked, helper pathways (18, 20, 22, 45–47). At this time we do not know if this will also be true for primary antibody responses in naive animals. If a separate subpopulation of Th cells is involved in the bystander help pathway, then this subpopulation is subject to different regulation than is that which supports cognate responses. This might explain the observation that cognate but not bystander responses are accelerated in adoptive recipients reconstituted with primed T cells. The putative T cell subpopulation responsible for bystander help may not be expanded after immunization or may be influenced by some extrinsic and independent form of regulation. Whatever the mechanism, the inability to generate bystander responses with secondary kinetics may reflect a form of regulation that is invoked to limit the extent of potentially deleterious nonspecific help.

The bystander pathway appears to be of general significance in primary, T cell-dependent antibody responses. The only constraint on the coimmunogen is its relative immunogenicity in a given animal. Our data suggest that, in addition, a wide variety of proteins may serve as indicator antigens. However, bystander help can only be detected *in vivo* under experimental conditions in which the indicator antigen lacks immunogenicity. In all cases that we have examined, H-2-linked genetic control of antibody responses can be bypassed by coimmunization. Furthermore, this generalization can be extended to include nonimmunogenic self antigens since Fl-specific antibody is induced after immunization with a mixture containing Fl-mouse hemoglobin and FGG. More importantly, the self antigen need not be modified by haptentation, since autoantibodies can be stimulated by coimmunization with unmodified mouse hemoglobin plus FGG. These observations raise the possibility that bystander help may be a mechanism that is responsible for the generation of autoantibodies that are produced during inflammatory responses (44). We envision that Th cells activated by microbial infection, for example, could support the differentiation of B cells recognizing the self antigens released after local cell destruction. A further understanding of the regulation of nonspecific help may provide insight into the induction of autoimmunity and the interaction of T cells and B cells during primary immune responses *in vivo*.

Summary

We evaluated the requirement for hapten-carrier linkage in the primary, T cell-dependent antibody response in vivo. Mice immunized with mixtures containing nonimmunogenic and immunogenic proteins developed antibody that was specific for determinants present on the nonimmunogenic carrier. Therefore, hapten-carrier linkage was not necessary for the generation of primary antibody responses. The magnitude of the bystander response was a function of the immunogenicity of the coimmunogen and the quantity of determinant-specific B cells available for activation. Interestingly, the kinetics of the bystander response, in contrast to the cognate response, were not accelerated in the presence of primed Th cells. Adoptive recipients reconstituted with primed Th cells developed accelerated cognate but not bystander antibody response, as compared with unprimed recipients. This phenomenon may reflect a regulatory mechanism invoked to limit the potentially harmful effects of nonspecific help. It was observed that while animals are tolerant to immunization with mouse (self) hemoglobin, immunization with a mixture containing mouse hemoglobin plus fowl gamma globulin resulted in the production of hemoglobin-binding autoantibodies. Thus bystander help induced by coimmunization may serve as a model for the induction of autoantibodies during normal immune responses in vivo.

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