Evidence for an immunoglobulin-dependent antigen-specific helper T cell

(immunoglobulin/lymphocyte receptors/agammaglobulinemia)

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ABSTRACT Evidence from various systems suggests that thymus-derived lymphocytes can affect the quality of antibody responses by recognizing various portions of the immunoglobulin receptor of bone-marrow-derived thymus-independent lymphocytes. A model for this process is proposed involving two antigen-specific mature T helper cells, one of which also is specific for immunoglobulin determinants. These two cells act synergistically. Evidence from adoptive secondary antibody responses demonstrates that both cells are antigen-specific T cells and that the immunoglobulin-recognizing T helper cell is absent from experimentally agammaglobulinemic mice. This cell is termed an "immunoglobulin-dependent T cell" because its activation requires the presence of immunoglobulin.

Antibody responses to most antigens involve the cooperation of thymus-independent lymphocytes (B cells) and thymus-dependent lymphocytes (T cells). Anti-hapten antibody responses to hapten-protein carrier conjugates involve antibody production by hapten-specific B cells; this response requires the cooperation of carrier-specific "helper" T cells (1-3). Although it has long been accepted that helper T cells must have specificity for carrier antigenic determinants, recently such cells have also been shown to recognize determinants encoded in the I region of the animal's own major histocompatibility complex (4-6). Even more recently, evidence from many experimental systems has suggested that helper T cells may also recognize the Ig determinants of B cells during this interaction (6-12). Evidence for these various types of recognition by T cells was recently summarized by Paul and Benacerraf (6).

The ability of T cells to recognize B cell Ig determinants has been demonstrated by examining the quality of the antibody response. In this way, it has been shown that helper T cells can affect the class or isotype of the antibody produced (7), its allotype (8), its charge (9, 10), its affinity (11), and its idiotype (12).

If helper T cells comprise but a single class of cells, the above evidence would suggest that they are able to recognize at least three distinct moieties on the B cell surface during cell cooperation: antigen, Ig, and I-region encoded markers. However, we have previously presented data suggesting that helper T cells, as commonly prepared by *in vivo* immunization, consist of at least two synergizing, carrier-specific populations (3, 13, 14). In this report we propose that both of these putative helper cells are mature, carrier-primed T cells and, furthermore, that one of them is dependent for its activation on the presence of Ig during priming. Evidence to support both of these points will be presented. Thus, we contend that one helper T cell recognizes antigen in association with I-region encoded structures, and the other recognizes antigen in association with Ig. The two

cells act synergistically by delivering activating signals to the same B cell.

The primary method of analysis we have used is measurement of the activity of limiting doses of carrier-primed spleen helper cells as determined from the anti-hapten antibody responses of irradiated recipient mice given a fixed number of hapten-primed B cells. In previous publications (3, 13, 14), the following findings were presented. When the logarithm of antibody response is plotted against the logarithm of helper cell number transferred, straight lines are obtained. At times early after boosting, when the titer of antibody is *increasing* rapidly, the slope of such lines is significantly greater than 1 (1.67 ± 0.05). A single, noninteracting population of cells should give slopes close to 1. Thus, these results suggest, but cannot prove, that there are two synergizing populations of helper cells in carrier-primed spleen. Another way to state this is that doubling the number of helper cells gives rise to nearly 4 times as much antibody. Two other points about the slopes of these lines are relevant. First, they tend to decrease with time after boosting, suggesting a feedback regulation of the response (suppression); however, in attempting to analyze interactions relevant to the induction of the response, it is the slope of the line prior to the onset of these regulatory phenomena that is important. Second, if carrier-primed helper spleen cells are held *constant* and hapten-primed spleen cells are titrated, the logarithmic plots yield straight lines with slopes close to 1, as expected for a single population of hapten-specific B cells.

To return to the helper cell titrations, various different treatments of this population altered its total helper activity, but in no case was the slope of the titration curve changed. Helper activity was resistant to adult thymectomy but highly sensitive to antithymocyte serum treatment of the donor, anti-Thy-1.2 and complement, and *in vitro* x-irradiation. Thus, both cells have the characteristics of mature T cells (14). Furthermore, the slope remained constant from 7 days after priming, when helper activity first appeared, to as long as 100 days after priming. The slope was not affected by titrating the antibody at higher hapten concentration, demonstrating that this phenomenon was not due to affinity differences in antibody responses at different levels of helper T cells. And this behavior of helper cells was seen with both lymph node and spleen helper cells. Finally, because the addition of excess spleen cells primed to an unrelated carrier, or of normal spleen cells, did not affect the slope or position of the titration curve, it was concluded that both of the helper activities were carrier-specific and dependent upon immunization.

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Abbreviations: B cells, thymus-independent bone-marrow-derived lymphocytes; T cells, thymus-dependent lymphocytes; Dnp, 2,4-dinitrophenyl; FITC, fluorescein isothiocyanate.

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To prove that we were indeed dealing with two carrier-specific; synergizing helper T cells, two further findings are necessary. Both are presented here. The first is that purified spleen T cells give a slope greater than 1. The second is to find a method for depleting at least one of the activities, obtaining a slope of 1, and then showing synergy when such depleted cells are added to carrier-primed spleen cells from normal donors. The latter was accomplished by raising helper T cells in agammaglobulinemic mice prepared by treatment with anti-mouse μ chain antibody from birth. This finding further suggested that the depleted population required Ig for its priming and allowed the synthesis of these data with the finding that T cells regulate the quality of the antibody response.

MATERIALS AND METHODS

Mice. BALB/cAnN mice were obtained from the Small Animal Section, Division of Research Services, National Institutes of Health. CBA/J mice were kindly provided by Bent Rubin, Statens Seruminstitut, Copenhagen, and CBA/H mice were bred at the Institute of Genetics, Karolinska Institute, Stockholm.

Antigens. Ovalbumin, keyhole limpet hemocyanin, and their 2,4-dinitrophenyl conjugates were prepared as described (3, 15).

Antisera. Rabbit anti-mouse μ chain antisera were prepared as described by Murgita *et al.* (16) and goat anti-mouse μ chain antibodies were prepared as described by Lawton *et al.* (17) or by Weinbaum *et al.* (18). Anti-Thy-1.2 was prepared and used as described (3). Guinea pig serum absorbed with mouse liver was used as a source of complement.

Fractionation of Spleen Cells on Ig-Anti-Ig Columns. Mouse Ig-rabbit-anti-mouse-Ig columns were prepared using Degalan beads as a supporting matrix, precisely as described by Wigzell (19). Columns were run at 4°.

Treatment of Mice with Anti- μ Chain Antibody. Three experiments were carried out with three different regimens. In the first, BALB/c newborn germ-free mice (Laboratory of Germ-Free Animal Research, National Institutes of Health) were injected repeatedly with normal goat IgG or purified goat anti- μ chain antibodies (17). In the second, conventionally raised mice were used and were treated with goat anti- μ chain globulin (18). In the third experiment, CBA/H mice were injected from birth with rabbit anti- μ chain serum according to Murgita *et al.* (16). The mice were immunized at 6 weeks of age and tested 1 week later.

Immunization. Spleen cell donors were primed with 10 μ g of ovalbumin or 100 μ g of Dnp₆-hemocyanin as described (15).

Adoptive Transfers. These were carried out as described (3, 13–15). The B cell source was spleen cells from mice primed with Dnp₆-hemocyanin and treated with anti-Thy-1.2 and complement before transfer. The helper cells were from ovalbumin-primed mice. Cell counts represent viable cells only. Mice were boosted with 5 μ g of Dnp₆-ovalbumin given intraperitoneally in saline within a day of transfer.

Antibody Assay. Mice were bled early (5–7 days) and at peak antibody titer (11–12 days) after boosting, and anti-Dnp antibody was measured by a modified Farr assay (3, 15). This assay measures antibody as the antigen-binding capacity of a serum, which is defined as the reciprocal of the dilution of antibody required to bind 33% of the ligand ϵ -Dnp-L-lysine at a free ligand concentration of 3.3 nM times the concentration of the free ligand. Data are plotted as the logarithm of the geometric mean antigen-binding capacity versus logarithm of cell number transferred. The slope of these lines is calculated by linear re-

gression analysis (3). Although generally only three points are presented, titrations with four points have confirmed that these are straight lines. However, on occasion, use of too few helper cells or too many helper cells for the number of B cells transferred leads to flattening of the curves at the lower or upper end, respectively.

Fluorescent Antibody Labeling of Cell Surfaces. The numbers of T and B cells were determined by surface staining with fluorescein isothiocyanate (FITC) conjugates of AKR anti-C3H thymocyte (anti-Thy-1.2) or goat anti-mouse Ig antibodies, respectively. The FITC-anti-Thy-1.2 was prepared from serum obtained by boosting mice with 10⁸ C3H thymocytes 3 months after the standard primary immunization against Thy-1.2 (3). They were bled 7, 9, and 11 days later. Standard procedures were used to conjugate the Ig fractions of these antisera with FITC, and they were purified by subsequent passage over DEAE-cellulose. The FITC-anti-Thy-1.2 gives smooth ring fluorescence with virtually all CBA thymocytes, with about 50% of CBA spleen cells, and with none of the AKR spleen cells. Staining with FITC-anti-Thy-1.2 is optimal if carried out at 37°.

RESULTS

Helper Activity of Purified Spleen T Cells from Carrier-Primed Mice. The experiment illustrated in Fig. 1 involved the transfer of a fixed number of Dnp-primed B cells plus various numbers of carrier-primed spleen cells. In Fig. 1A, representing titers 7 days after boosting, the slope of the plot for unseparated spleen cells is 2.3, suggesting two interacting cells in the carrier-primed spleen. That both cells are T cells is shown in the other two curves. One represents the helper activity of spleen T cells prepared by passage over an Ig-anti-Ig column. The slope of this line is 1.9, and the activity per cell is increased 2- to 3-fold. Furthermore, the addition at each point of 2.5 times as many anti-Thy-1.2 and complement-treated ovalbumin-primed spleen cells (B cells) did not affect the activity of the purified T cells, suggesting that their helper activity is an expression of T cells alone. Fig. 1B shows these data versus T cell number transferred, as determined from cell surface labeling. Per T cell transferred, all preparations had nearly equal activity. Fig. 1C shows the response at peak antibody titer. Here, the slopes decreased to 1.2–1.5, and again all preparations had similar activity per T cell. This experiment was repeated twice, with slopes of 1.7 and 2.4 at day 7 for the purified T cells.

Helper Activity of Carrier-Primed Spleen Cells from Mice Treated from Birth with Anti- μ Chain Antibodies. We carried out three similar adoptive transfer experiments using carrier-primed cells from mice made agammaglobulinemic by treatment with heterologous anti-mouse μ chain serum from birth. The results of one such experiment are given in Fig. 2, and a summary of all three is presented in Table 1. Spleen cells from anti- μ treated mice gave a decreased slope (0.80) early after

Table 1. Slope of logarithm of antibody response versus logarithm of number of helper cells transferred at early and late bleedings

Helper cell donor pretreatment	Slope at various times after boosting*	
	Days 5–7	Days 11–12
Control	1.50; 1.67; 1.33	0.96; 1.35
Anti- μ treated	0.84; 0.80; 0.83	0.84; 1.09

* Values for three different experiments at days 5–7 and for two of these at days 11–12; third experiment was not titrated at days 11–12.

FIG. 1. Anti-Dnp antibody responses (antigen-binding capacity $\times 10^{-8}$ M) of irradiated CBA/J mice given 22×10^6 Dnp-primed B cells plus varying numbers of helper cells primed with ovalbumin and boosted with Dnp₈-ovalbumin. (A and B) Tested 7 days after boosting. (C) Tested 12 days after boosting. O, Unfractionated spleen cells, 37% Ig-positive; □, spleen T cells purified over an Ig-anti-Ig column, 1.5% Ig-positive, 2-fold enriched for Thy-1.2-positive cells; ▲, anti-Thy-1.2- and complement-treated spleen cells; △, spleen T cells plus 2.5 times as many anti-Thy-1.2- and complement-treated spleen cells. Note different scale in C.

boosting (Fig. 2A), compared to cells from littermate controls (slope = 1.67). However, at peak titer, the two populations did not differ significantly in slope or activity (Fig. 2B). In this experiment, the spleen cells from anti- μ treated mice contained no cells with surface Ig, whereas control mice had 30% surface Ig-positive cells. The number of T cells in these preparations was not determined, but in other experiments with anti- μ treated mice it has been shown that they contain increased numbers of T cells [up to 2-fold more than in controls (17)], so that if the data were plotted per T cell transferred, the line for control spleen cells would be shifted about 2-fold to the left. The finding of a decreased slope in titrations of helper cells from anti- μ treated mice was reproducible in three experiments, as shown in Table 1.

Table 2 shows that transferring a mixture of primed spleen cells from anti- μ treated mice and primed spleen cells from control mice gave responses greater than or equal to that predicted from the sum of the responses given by each cell population transferred alone. This is important for two reasons. First, it makes suppression unlikely as the mechanism responsible for lowering the helper activity of spleen cells from anti- μ treated mice. Second, it suggests that helper spleen cells from normal mice contain cells that significantly increase the helper activity of spleen cells from anti- μ treated mice.

DISCUSSION

The data presented here strongly support the hypothesis put forward in the introduction to this paper: that optimal help for B cell responses to antigen involves the synergistic action of two types of carrier-specific mature helper T cells that differ in that

one recognizes antigen in association with Ig molecules and the other presumably recognizes antigen in association with I-region encoded structures (4-6). Their synergistic interaction probably takes place on the surface of a hapten-specific B cell to which the hapten-carrier complex is bound via the B cell's intrinsic Ig receptor. On the other hand, priming of each of these classes of helper T cells probably occurs at the surface of an antigen-binding macrophage. Both B cells and macrophages bear surface Ig and I-region encoded structures.

Table 2. Helper activity of mixtures of spleen cells from carrier-primed control and anti- μ treated donors

Helper cells transferred, no. $\times 10^{-6}$		Anti-Dnp antibody response, antigen binding capacity $\times 10^{-8}$ M			
		Alone		Mixed	
Control	Anti- μ treated	Control	Anti- μ treated	Predicted*	Found
Day 5					
3.8	4.5	0.7	1.1	1.8	5.3
12.5	15	5.1	2.9	8.0	10.7
Day 12					
3.8	4.5	50.4	42.5	92.9	244
12.5	15	215	200	415	337

* Predicted values were determined by addition of the antibody response given by that number of helper cells given alone. That is, 3.8×10^6 control cells at day 5 give an antigen binding capacity of 0.7 and 4.5×10^6 anti- μ treated cells give 1.1 units, so both transferred together, providing that they do not synergize, should give 1.8.

FIG. 2. Anti-Dnp antibody responses (antigen-binding capacity $\times 10^{-8}$ M) of irradiated BALB/c mice given 20×10^6 Dnp-primed B cells plus varying numbers of helper cells primed with ovalbumin and boosted with Dnp₈-ovalbumin. (A) Tested 5 days after boosting. (B) Tested 11 days after boosting. O, Spleen cells from mice treated with anti- μ chain serum from birth; Δ , spleen cells from littermate control mice. Note different scale in B.

An alternative explanation for the results in experiments using anti- μ treated mice would be that T cells express surface Ig molecules, either actively synthesized or passively acquired. This seems unlikely, because spleen cells passed through Ig-anti-Ig columns to remove surface Ig-bearing cells did not differ in slope or activity per T cell from the spleen population from which they were derived.

The data with anti- μ treated mice are analogous to those of other investigators using different systems. Herzenberg *et al.* (8) treated mice with anti-allotype antibody at birth, and such mice lost the ability to produce T helper cells for antibody of that allotype. This is also true of helper cells from mice lacking the same allotype for genetic reasons (8). Furthermore, when they eliminated the allotype-specific help from a carrier-primed spleen T cell population and then added that population to a small number of untreated carrier-primed helper T cells, more help for allotype than predicted was found (see bottom line of table 6 in ref. 8). This finding is similar to that in Table 2 of this paper.

Another example is the recent work of Ward and Cantor (12), who blocked the immunization of helper cells for an inherited antiarsonate idiotypic (20) by treating the helper donors with anti-idiotypic shortly before carrier priming. Again, adding such idiotypic-negative helper T cells to helper T cells from non-suppressed, carrier-primed spleen cells led to a significantly greater production of idiotypic antibody than would be predicted by addition of the idiotypic antibody response given by either population transferred alone. Thus, when helper T cell

donor mice lack Ig (these experiments), the entire response is reduced; when they lack an allotype, the antibody response of that allotype is reduced; and when they lack an idiotypic, the response of that idiotypic is reduced. In all cases, adding the defective T cells to carrier-primed T cells from control mice leads to synergistic increases in just that portion of the response that has been eliminated by anti-Ig treatment of the T helper cell donor.

Several points need further clarification. We have only indirect evidence that both T helper cells are carrier-specific. It is possible that the Ig-dependent T helper cell is specific only for Ig, and that it arises in response to the Ig production induced by carrier priming. This seems unlikely, because both activities are fully present 7 days after priming and remain essentially unchanged for at least 100 days. However, it can be tested by combining helper cells from anti- μ treated mice primed to one carrier with helper spleen cells from control mice primed with a second carrier and testing for synergy. This can be more precisely tested in the system described by Ward and Cantor (12), in which production of the idiotypic in question is not induced by carrier priming and in which any carrier can lead to the induction of idiotypic formation, suggesting that the T helper cell specific for the idiotypic does not arise solely in response to Ig or idiotypic production induced by carrier priming.

A second experimental goal is to deplete the other helper T cell activity, which we believe is specific for antigen seen in association with I-region encoded structures. This might be done

by educating T cells in the presence of one major histocompatibility complex and testing them with B cells of a different one. Cells so tested might synergize with helper cells derived from anti- μ treated mice. Also, one might attempt to reconstitute the defect in anti- μ treated mice with passive antibody or with antibody adsorbed to macrophages. Finally, it would be of value to type both T helper cells for surface Ly antigens and for the presence of Ia antigenic specificities. Because Ward and Cantor (12) used anti-Ly-2,3-treated T cells in their experiments, we would expect both cells to be Ly-1⁺, Ly-2,3⁻. This is especially so because these authors found parabolic dose-response curves with Ly-1⁺, Ly-2,3⁻ helper cells; parabolic curves become straight lines with slopes around 2 when logarithm-transformed. Also, if one of these two T helper cells were Ia positive and the other Ia negative, it might explain some of the confusion about the Ia antigen status of helper T cells (21).

Underlying our hypothesis is the notion that T cells respond to antigen in association with a second, self-derived, cell surface marker. Those that have so far been clearly defined are Ig, I-region encoded structures, and the classical serologically defined histocompatibility antigens called K and D in the mouse. These arguments have been summarized in a number of recent papers and possible explanations for these findings have been proposed (6, 22-24). By suggesting that the regulation of the quality of the antibody response is a function of a class of T cells specific for the antigen seen in association with Ig and that these cells are distinct from, and can act synergistically with, T cells specific for antigen seen in association with I-region encoded structures, the number of distinct specificities per T cell can be held to two (or one, if one argues that a single receptor recognizes the specific complex of antigen plus Ig or I-region encoded structure). This hypothesis would also explain our finding, which was previously puzzling to us, that there appear to be two synergizing, mature, carrier-specific, helper T cells in carrier-primed spleen (3).

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