INDIVIDUAL ANTIGEN-SPECIFIC T LYMPHOCYTES: HELPER FUNCTION IN ENABLING THE EXPRESSION OF MULTIPLE ANTIBODY ISOTYPES*

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Since the demonstration that antigen-specific T lymphocytes play a role in the promotion of antibody responses of B lymphocytes (1, 2), much effort has been directed towards precisely defining the specificity and functional capabilities of "helper" T cells in T-dependent antibody responses. The collaborative function of T cells appears to be dependent upon the recognition of antigen, a process which under certain circumstances may be highly specific (3, 4), as well as upon the recognition of the cell surface products of the major histocompatibility complex on syngeneic B cells or macrophages (5–7). However, the exact mechanism of T-cell–B-cell collaboration and the specific nature of the T cells involved are still poorly understood. This is due in part to the fact that T-cell populations are generally complex mixtures of cells which differ not only in antigen specificity but also in the functions which they exhibit after appropriate contact with antigen. T cells have been shown to be capable of mediating a number of diverse functions which serve to both enhance and suppress antibody responses (1, 2, 8). Since little is known about the stimulatory conditions which induce these diverse T-cell functions, any observed immune phenomenon may reflect the composite activity of several T cells (both helper and suppressor), rather than the function or capability of an individual T cell.

Among the most provocative roles attributed to helper T-cell collaboration in humoral responses is the promotion of the synthesis of multiple Ig isotypes by B cells (9, 10). It has been demonstrated previously in this laboratory that individual hapten-specific primary B cells yield clones which produce multiple Ig isotypes when stimulated in the presence of excess syngeneic, carrier-specific T cells (6, 11, 12). The experimental evidence available at this time cannot delineate whether or not more than a single T cell is required for the expression of multiple Ig isotypes (13, 14). Several studies, however, have implied that individual T cells (13) or selected T-cell subpopulations (15, 16) may be restricted to enabling B-cell antibody responses of only a single Ig isotype or allotype.

An unambiguous evaluation of the potential of individual T cells to enable multiple isotype expression by one or several B lymphocytes requires an analysis of individual T cells in the presence of excess antigen-specific primary B cells. Other investigators have used limiting numbers of T cells to estimate the frequency of alloreactive T cells (17, 18), and to analyze various aspects of helper T-cell function, such as allogeneic collaboration (19) or multiple B-cell stimulation (13).

In this report we describe an experimental system which allows the isolation and stimulation of individual antigen-specific helper T cells in a splenic environment which provides an excess of primary B cells for collaboration with the isolated T lymphocyte. This has been accomplished by employing much of the technology and

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methodology of the B-cell splenic focus analysis developed to study individual B-cell antibody responses in vitro (6, 12, 13, 20, 21). Employing this system, we have demonstrated than an individual T cell, specific for the PR8 strain of influenza virus, has the capacity to enable primary B-cell PR8-specific antibody responses of more than a single Ig isotype.

Materials and Methods

Antigen and Immunoadsorbents. The growth and preparation of the A strain influenza virus, PR8 (A/PR8/34(HON 1)), has been described previously (21, 22). *Limulus polyphemus* hemocyanin (Hy) was purchased from Worthington Biochemical Corp., Freehold, N. J., and ovalbumin (OVA)[1] was obtained from Sigma Chemical Co., St. Louis, Mo.

Animals and Immunizations. B10.D2 mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. B10.D2 mice are a congenic resistant strain that shares only genes of the H-2 complex with BALB/c recipients. Previous findings have indicated that syngeny at H-2 maximizes T-cell–B-cell collaboration (5–7), and that allogeny at allotype (B10.D2 are a^2, whereas BALB/c are a^1) circumvents an antibody-specific suppression of primary B-cell responses (23). Athymic nu+/nu+ BALB/c mice were purchased from ARS Sprague-Dawley, Madison, Wis. The nude gene was transferred with a cross-intercross-backcross system to the BALB/c inbred strain.

Mice to be used as T-cell donors were immunized intraperitoneally with 200 hemagglutinating units of PR8 in 0.2 ml of saline 4–6 wk before cell transfers.

T-Cell Transfer and Fragment Cultures. Single cell suspensions of mesenteric lymph nodes from PR8-immune B10.D2 mice were prepared in Dulbecco's phosphate-buffered saline (PBS) supplemented with 5% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) by pressing the lymph nodes between glass slides. Cell suspensions were then passed over nylon wool columns (Fenwal Laboratories, Inc., Deerfield, Ill.), according to the protocol of Julius et al. (24). Approximately 30% of the applied cells were recovered, and >95% of these cells were sensitive to anti-θ sera and complement treatment as determined by trypan blue exclusion. Cells were then transferred intravenously to nu+/nu+ BALB/c mice in 0.25 ml of Dulbecco's modified Eagle's medium. Recipient spleens were aseptically removed 48 h later, and diced to yield ≅50 1-mm^3 fragments. The fragments were individually cultured and stimulated with PR8 as described previously (21, 22).

The supernates from fragment cultures were assayed for the presence of PR8-specific antibody and the immunoglobulin isotype of the PR8-specific antibody between days 9 and 16 of culture, using a solid-phase radioimmunoassay (RIA) which has been described elsewhere (11, 12, 21, 22).

Anti-θ Sera Treatment. The anti-θ sera used in these studies were the kind gift of Dr. Ross Basch (New York University School of Medicine). The anti-θ serum was obtained from rabbits immunized with mouse brain, and it was extensively adsorbed with mouse liver. This antisera and guinea pig complement killed >95% of mouse thymocytes as determined by trypan blue exclusion tests. The antisera had no cytotoxicity for mesenteric lymph node B cells, as determined by the unimpaired function of antigen-specific B cells in the splenic focus fragment culture system (20) after pretreatment of B-cell donor inoculum with the antisera and complement.

Single cell suspensions of donor lymph node cells that had been passed over nylon wool columns were incubated at 4°C with an appropriate dilution of the antisera in Dulbecco's PBS containing 5% heat-inactivated fetal calf serum (FCS). The cells were then washed twice in cold PBS-FCS and resuspended in PBS containing an appropriate dilution of lyophilized guinea pig complement (Grand Island Biological Co.). The cells were incubated for 45 min at 37°C in a 5% CO_2, 95% air atmosphere. Cell viability was determined by trypan blue exclusion. The cells were washed 4–6 times in Dulbecco's modified Eagle's medium before cell transfer.

Results

Limiting Dilution Analysis of Lymph Node Cells from PR8-Immunized Mice. The results

[1] *Abbreviations used in this paper:* FCS, fetal calf serum; Hy, *Limulus polyphemus* hemocyanin; OVA, ovalbumin; PBS, phosphate-buffered saline; RIA, radioimmunoassay.

obtained when graded numbers of column-enriched mesenteric lymph node cells from PR8-immunized donors were transferred to nu+/nu+ BALB/c mice are shown in Fig. 1. The percent of fragments that produced PR8-specific antibody is plotted against the number of donor lymph node cells transferred to each recipient mouse. PR8 has been previously demonstrated to be a T-dependent antigen for the stimulation of primary B lymphocytes (25). As shown in Fig. 1, when no donor cells were transferred, no anti-PR8 antibody was detected in the culture supernates of the recipient spleen fragments. Thus the response of nude BALB/c spleen cells to PR8 was totally dependent upon the presence of donor cells. As the number of PR8-specific donor lymph node cells transferred to the recipient was increased, the number of observed recipient spleen fragments which synthesized PR8-specific antibody increased linearly. When fewer than 0.8×10^6 donor cells were transferred, the linearity of this relationship provided the strongest possible evidence that only one cell type in the donor cell inoculum necessary for antibody synthesis was being titrated out in the recipient spleen fragments. As shown in Fig. 1, the percent positive spleen fragments at each cell dose closely follows that predicted by the Poisson distribution. Most responses, therefore, were presumably the function of individual donor helper T-cells in the recipient spleen fragments when between 0.1 and 0.8 donor T cells were transferred to recipients.

Treatment of the donor T cells with anti-θ serum and complement before cell transfer eliminated the PR8-specific response, even when as many as 1.0×10^6 anti-θ-treated donor cells were administered per recipient. Furthermore, total body irradiation (1,300 R) of recipient mice also eliminated the PR8-specific antibody response. Finally, donor cells prepared from the lymph nodes of either nonimmune mice or mice immunized with Hy or OVA failed to produce an anti-PR8 antibody response in the nude BALB/c spleen fragments. These results indicate that: (*a*) the nu+/nu+ BALB/c anti-PR8 antibody response was dependent upon sufficient numbers of transferred donor lymph node cells; (*b*) the response obtained was not due to the transfer of contaminant immune (secondary) B cells in the donor cell inoculum; and (*c*) the cell type in the donor cell preparation responsible for promoting the response is anti-θ-plus-complement sensitive, and is present only in PR8-immune animals. It is important to note that the donor cells were not irradiated before transfer, and they were therefore capable of proliferating in the spleen fragments. The antibody responses obtained may have resulted from the interaction of the recipient B cells with the clonal progeny of the isolated individual donor T cell.

Isotype Analysis of PR8-Specific Antibodies. The results of an analysis of the Ig heavy chain isotypes of the PR8-specific antibodies detected in the supernates from splenic fragments of nu+/nu+ BALB/c mice after T-cell transfer are shown in Table I. It is clear from these results that the majority of fragments which produced PR8-specific antibody synthesized more than a single heavy chain isotype, even when very few donor T-cells were transferred to the nude recipients. Since the distribution of donor cells in the nude BALB/c spleen fragments appeared to be random when 2.5×10^5 donor cells were transferred per recipient (Fig. 1), the Poisson distribution predicts that only 8.5% of the responding spleen fragments should have contained more than a single PR8-specific responding T cell. Yet greater than 50% of responding fragments produced antibodies of multiple heavy chain isotypes. This marked disparity, particularly in the high number of fragments producing IgM as well as either IgG_1 or IgA is consistent for all T-cell dilutions. Thus, it appears that a single antigen-specific T

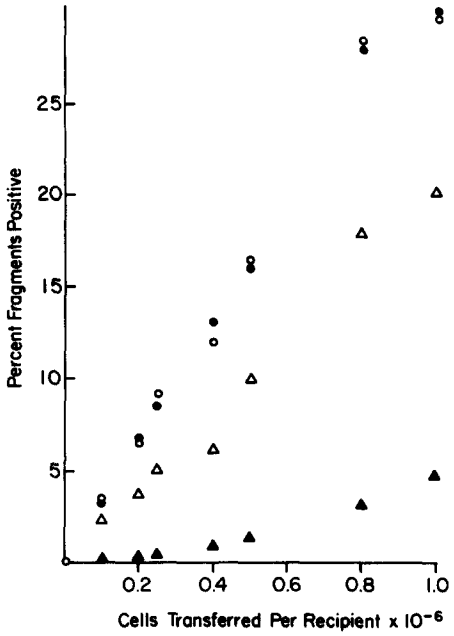

FIG. 1. The percent of spleen fragments producing anti-PR8 antibody is plotted versus the number of donor cells transferred to each recipient (○). The estimated percent of spleen fragments synthesizing anti-PR8 antibody (●) is calculated using the Poisson formula P (×) = $e^{-\mu}\mu^x/x!$, employing the experimentally derived mean proportion of responding fragments per 10^6 cells transferred at each cell dose to determine μ. The percent of spleen fragments synthesizing anti-PR8 antibody of more than a single heavy chain class (Δ) is also plotted. For comparative purposes, the Poisson prediction of the percent of spleen fragments containing more than a single antigen-specific T cell (▲) is plotted for each experimental point.x!, x factorial.

TABLE I

The Heavy Chain Isotype of Anti-PR8 Antibodies Produced by Spleen Fragments

No. donor cells transferred per recipient × 10^{-6}	Total no. positive fragments analyzed*	Percent of fragments that synthesized:‡						
		Single isotypes			Multiple isotypes			
		IgM	IgG_1	IgA	IgM + IgG_1	IgM + IgA	IgG_1 + IgA	IgM + IgG_1 + IgA
0.10	24	25	4	8	12	46	<1	4
0.25	20	30	15	<1	20	25	<1	10
0.50	23	17	9	13	4	48	4	5

* Positive anti-PR8 antibody-producing spleen fragments were detected using ^{125}I-labeled anti-mouse Fab in an RIA.

‡ These figures represent the percent of the total number of clones that synthesized IgM, IgG_1, or IgA alone, or combinations of these immunoglobulin isotypes as detected with ^{125}I-labeled goat anti-mouse μ-, γ_1-, and α-antibodies in an RIA (11,12).

cell is capable of helping B cells generate antibody responses of more than a single heavy chain isotype. It is not possible to determine from the results presented here whether one or several PR8-specific B cells were responsible for the synthesis of multiple heavy chain isotypes of PR8-specific antibody in the spleen fragments.

Discussion

The studies presented in this report have examined the capabilities of individual helper T lymphocytes to enable the production of antibodies of multiple Ig heavy chain isotypes in a T-dependent antibody response. Individual T cells were isolated by the transfer of PR8-sensitized, nylon wool column-enriched, lymph node T cells to recipient nu+/nu+ BALB/c mice. When fewer than 0.8×10^6 T cells were transferred, the number of recipient fragments that produced PR8-specific antibody was linearly related to the number of donor T cells transferred. The linearity of this relationship indicates that each antibody-producing fragment was the result of the presence and stimulation of a single PR8-specific donor T cell. Since as few as 10^5 transferred T cells yielded responding fragments, it is unlikely that a second T-cell type present in the donor cell inoculum in very high frequency participated in the generation of this response.

We have presented the results of an analysis of the heavy chain isotypes of the anti-PR8 antibody synthesized as a result of B-cell stimulation in collaboration with a single, antigen-specific donor T cell. These results indicate that the majority of antibody-producing spleen fragments synthesized antibody of more than a single heavy chain isotype. It should be pointed out that donor T lymphocytes may be capable of proliferating in the recipient splenic fragment such that antibody responses may have been due to B-cell collaboration with the clonal progeny of the donor T cell. In addition, these studies have not delineated whether the synthesis of multiple heavy chain isotypes represents the stimulation of a single PR8-specific B cell within the recipient spleen fragments, or of several independent PR8-specific B cells.

Previous reports from this laboratory have demonstrated that the clonal progeny of a single B cell had the potential to synthesize antibody of several heavy chain classes specific for the antigens 2,4-dinitrophenyl-Hy and phosphorylcholine-tyrosylglycyl-glycine-Hy (11, 12). In a subsequent report it was shown that the heavy chain class of the primary anti-DNP and anti-phosphorylcholine B-cell responses is, in part, dictated by the nature of the T-cell help, in that allogeneic T-cell–B-cell collaboration resulted in clones synthesizing only IgM antibody (6). Several investigators have demonstrated restrictions in the heavy chain isotype of antibody synthesized in T-dependent antibody responses using various immunization protocols, even under syngeneic conditions (13, 15, 16). Recently, a great deal of significance has been attributed to one interpretation of these findings—that individual T cells may be restricted to collaborating with B cells of only a single allotype (16) and, by analogy, perhaps a single idiotype (26). The results presented in this report directly demonstrate that for primary B-cell responses, the vast majority of PR8-specific T cells are neither isotype- nor, by inference, allotype-restricted. Indeed, donor T cells in the system used were allogeneic to responding B cells at the heavy chain allotype locus.

For this investigation we have chosen an experimental system which may have maximized the potential for fruitful collaborative interactions by antigen-specific T cells. Each fragment contained as many as 100 PR8-specific primary B cells for collaboration with any lodged T cell as calculated on the basis of the total frequency of PR8-specific B cells per spleen (25). It is possible that a resident T cell may have selectively enabled multiple isotype expression by only one or very few of these B cells. For example, multiple heavy chain isotype expression may require an appropriate selective matching based on cell-cell recognition of individual T and B lympho-

cytes, and such matching might be facilitated when primary B cells are in great excess. In addition, PR8 is a particulate, complex antigen which may also facilitate T-cell–B-cell interactions. In this context, it should be noted that previous investigations using 2,4,6-trinitrophenyl-keyhole limpet hemocyanin showed that most T cells may collaborate with only a single B cell (13), whereas isolated sheep erythrocyte-specific T cells may collaborate with many B cells (2, 14). Indeed, our own preliminary investigations indicate that isolated Hy- or OVA-specific T-cells yield antibody responses which are predominately of the IgM heavy chain isotype.

It is clear from the studies presented in this report that under appropriate conditions, individual T cells can enable primary B-cell responses of multiple Ig heavy chain isotypes. Future experiments should provide greater insights as to whether all T-cell–B-cell interactions can be equally fruitful, and what factors, including antigen form and cell-cell recognition, modulate the outcome of such interactions.

Summary

In recent years antigen-specific T cells have been shown to be capable of mediating a number of diverse functions in collaboration with B cells in humoral immune responses. One of the more intriguing roles attributed to helper T cells is the promotion of the synthesis of multiple immunoglobulin isotypes by B cells in T-dependent antibody responses. The experiments presented in this report were carried out to determine if an individual antigen-specific T lymphocyte has the capability to enable the production of antibodies of multiple immunoglobulin heavy chain isotypes. We describe an experimental system which allows for the isolation and antigenic stimulation of individual helper T cells in a splenic environment which provides an excess of primary B cells for collaboration with isolated T lymphocytes. Employing this system we have demonstrated that an individual antigen-specific T lymphocyte, specific for the PR8 strain of influenza virus, has the capacity to enable primary B-cell PR8-specific antibody responses of more than a single immunoglobulin isotype. The implications made by these studies regarding the problem of genetic restrictions regulating T-cell–B-cell interaction is discussed.

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