T CELL REGULATION OF IgG SUBCLASS ANTIBODY PRODUCTION IN RESPONSE TO T-INDEPENDENT ANTIGENS

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Although thymus-matured T cells are not necessary for in vivo B cell responses to a class of antigens designated T-independent (TI)[1] (1, 2), we wished to determine whether T cells could influence the nature of the antibody produced. It was our hypothesis that in a TI antigen system in which there is no obligate need for carrier-specific helper T cell triggering signals, T cells may still exert subtle regulatory effects on the B cell antibody response via a distinct population of Ig-specific regulatory cells (3–9). If this were indeed the case, such a system might be ideal for studying the regulatory effects of Ig-specific T cells independently of carrier-specific T cells. The characteristics of Ig-specific T cell interactions with B cells could thus be elucidated and compared with the known characteristics of carrier-specific T cell-B cell interactions.

In testing for T cell influences on B cell responses to TI antigens, our emphasis has been to search for possible effects of isotype-specific T cells. Our approach has been to determine whether T cells could differentially regulate the levels of the various isotypes of antibody made by the B cell population responding to a subset of TI antigens, designated type 2 TI (TI-2) antigens. These antigens are characterized by their lack of polyclonal activating activity and their inability to stimulate B cells from neonatal mice or mice with the CBA/N immune defect (10). We here demonstrate that the in vivo B cell response to TI-2 antigens can be influenced by T lymphocytes and that this influence is restricted to only some of the Ig isotypes produced, namely the IgG2a, and, to a lesser extent, the IgG2b subclasses.

Materials and Methods

Animals. C57BL10/ScN *nu/nu*, C57BL10/ScN *nu/+*, C57BL/KaLwN, and (CBA/N × C57BL/6)F_1 mice were obtained from the Small Animal Section, Division of Research Services, National Institutes of Health, Bethesda, Md. Age matched *nu/nu* and *nu/+* male and female mice from 2–4 mo of age were used in each experiment.

Antigens and Immunizations. Trinitrophenyl-aminoethylcarbamylmethyl (TNP-AECM)-Ficoll (mol wt = 400,000; 55 mol TNP/mol Ficoll) was purchased from Biosearch (San Rafael, Calif.). TNP-AECM-Levan, a gift of Dr. Constantin Bona, National Institutes of Health, Bethesda, Md., was prepared as previously described (11). Mice were immunized with 10 μg antigen intraperitoneally. Animals were bled 1 d before immunization and from 5–52 d after

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[1] *Abbreviations used in this paper:* AECM, aminoethylcarbamethyl; BSA, bovine serum albumin; C, guinea pig complement; CFA, complete Freund's adjuvant; G, goat; PBS, phosphate-buffered saline; TI, T-independent; TI-2, type-2 T-independent; TNP, trinitrophenyl.

immunization. Pools of serum consisted of equal aliquots taken from the individual serum samples within a group. Experimental groups consisted of 3–10 mice.

Selective Enrichment for B or T Cell Populations. To enrich selectively for B cells, *nu/+* and *nu/nu* spleen cell populations were processed in the following manner. Red blood cells and dead cells were removed by sequential treatment with lysing buffer (12) and low ionic strength buffer (13). Cells were then treated with a 10^{-4} dilution of a monoclonal anti-Thy-1.2 antibody (New England Nuclear Corp., Boston, Mass.) at a cell concentration of 5×10^6/ml for 45 min at room temperature, centrifuged, and further incubated with a 1:15 dilution of guinea pig complement (C) (Flow Laboratories, Inc., Rockville, Md.) for 45 min at 37°C. The anti-Thy-1.2 and C treatment was repeated. Dead cells were removed by reexposure to low ionic strength buffer. In a typical experiment, treated cells from *nu/nu* mice were 78% Ig^+, whereas those from *nu/+* mice were 82% Ig^+ by fluorescence microscopy.

Spleen cell suspensions were selectively enriched for T cells by the method described by Mage et al. (14). After two goat anti-Ig-coated plate passages, the resulting nonadherent cells ranged from 1–3% Ig^+ and 83–95% Thy-1^+. Cell recovery in nonadherent fraction ranged from 20–30%.

Radioimmunoassay for Anti-TNP Antibody. Flexible polyvinyl chloride microtiter plates (Dynatech Laboratories, Inc., Dynatech Corp., Alexandria, Va.) were coated with 10 μg/ml TNP bovine serum albumin (BSA) in phosphate-buffered saline (PBS), pH 7.4 (50 μl/well). TNP-BSA was prepared according to the method of Rittenberg and Amkraut (15) and contained 20 mol of TNP/mol of BSA. After 1 h, plates were washed and coated with 50% fetal calf serum (North American Biologicals, Inc., Miami, Fla.) to prevent further nonspecific binding (100 μl/well). After a further 1 h incubation, the plates were washed four times and 50 μl of the appropriate serum diluted in assay buffer (PBS containing 1% BSA + 0.1% sodium azide + 0.005 M disodium ethylenediamine tetraacetate) was added. Anti-TNP serum dilutions were incubated in the wells for 3 h after which the wells were washed six times with PBS, and 50 μl of the appropriate ^3H-labeled antibody (described below) was added in assay buffer to each well. After an additional 3-h incubation, the labeled antibody was removed, the plates were washed 13 times with tap water, and the wells were subsequently separated by slicing off the top of the microtiter plate. Each well was placed in scintillation fluid and measurements of the amount of bound ^3H-labeled antibody were made in a liquid scintillation spectrometer.

Anti-TNP sera were tested at 8 or 12 twofold dilutions. When log counts per minute bound was plotted against the log of serum dilution, a curve with a linear region was obtained. This linear region generally included the point at which 1% of added ^3H anti-Ig was bound. The serum dilution that bound 1% was obtained from the curve and this value was reported as the antibody titer.

Myeloma Proteins used to Purify and Test Specificity of Anti-Isotype Antibodies. Myeloma proteins were purified from ascitic fluids of mice bearing MOPC-21, MOPC-245, UPC-10, CBPC-101, MOPC-195S, and BPC-4 plasmacytomas [respective class and allotype designations (16) are IgG1, Igh-4a; IgG1, Igh-4b; IgG2a, Igh-1a; IgG2a, Igh-1b; IgG2b, Igh-3a; and IgG2b, Igh-3b] by a protein-A Sepharose pH gradient elution technique (17). TEPC-183, MOPC-104E, TEPC-15, FLOPC-21, and J606 purified Igh^a myeloma proteins (IgM, IgM, IgA, IgG3, and IgG3, respectively) were commercially obtained (Litton Bionetics, Kensington, Md.). Purified myeloma proteins representative of the various isotypes were coupled to cyanogen-bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) and used as immunoadsorbents.

Preparation of Isotype-Specific Antibodies. Sera from goats (G) immunized with Fc fragments from MOPC-31C (Igh-4a), RPC-24B (Igh-1a), CBPC-101 (Igh-1b), or MPC-37 (Igh-3a) in complete Freund's adjuvant (CFA) and from rabbits immunized with FLOPC-21 (IgG3) in CFA were rendered subclass-specific by adsorption on Sepharose columns coupled with myelomas of other isotypes. They were affinity purified using columns containing myeloma proteins of the isotype used for immunization. Affinity-purified G anti-IgM was a gift of Dr. Donna Sieckmann (Naval Medical Research Institute, Bethesda, Md.). Affinity-purified antibodies at a concentration of 0.5–1.5 mg/ml were tritiated with NaB^3H_4 (sp act 40–50 C/mM; New England Nuclear) by the reductive methylation procedure of Wilder et al. (18). ^3H-labeled antibodies had specific activities ranging from $0.5–2.6 \times 10^6$ cpm/μg. They were diluted in

PBS + 2% BSA + 0.1% sodium azide, and stored in liquid nitrogen for up to 1 yr without noticeable loss of activity.

Analysis of Specificity of ^{3}H-labeled Anti-Isotype Reagents. Tests for specificity were done by measuring the direct binding of ^{3}H-labeled reagents to microtiter wells precoated with purified myeloma proteins representative of the various isotypes. In the case of the IgG1, IgG2a, and IgG2b subclasses, binding to myeloma proteins of both the Igh^a and Igh^b allotypic forms was measured. In addition, inhibition assays were performed to compare the ability of various isotypic forms of the myeloma proteins to inhibit the binding of ^{3}H-labeled antibodies to plate-bound myeloma proteins. Through the combination of these approaches, the ^{3}H-labeled anti-isotype reagents were ascertained to have specificity for the isotype used for immunization. The G anti-CBPC-101 reagent had specificity for the Igh-1^b allotypic form of IgG2a.

Comparison of the Binding Efficiencies of the Various ^{3}H-labeled Anti-Isotype Antibodies. To validate the comparison of anti-TNP-antibody titers of one isotype versus another, the binding efficiencies of the various labeled antibodies were compared in terms of counts per minute bound to a known quantity of a given isotype. Wells were coated with affinity-purified rabbit anti-kappa light chain antibody. After washing, saturating nonspecific binding sites with 1% BSA in assay buffer, and washing again, ^{3}H-labeled myeloma proteins were added to the wells, the percent counts per minute bound was determined, and the absolute amount of myeloma (in nanograms) bound to the rabbit anti-kappa coated wells was then calculated. The amount of tritiated myeloma protein chosen for addition in these experiments was determined by prior studies to be in the linear portion of a dose-response curve relating amount of myeloma protein added and amount of tritiated anti-Ig antibody bound. Specific activities of TEPC-183, MOPC-21, CBPC-101, MOPC-195S, and FLOPC-21 labeled proteins were 2.0×10^6, 1.7×10^6, 2.0×10^6, 1.9×10^6, and 2.5×10^6 cpm/μg, respectively. To some of the wells that had bound ^{3}H myeloma·protein, ^{3}H-labeled anti-isotype antibody (40,000–70,000 cpm) was added. The percent ^{3}H-labeled anti-isotype antibody bound per nanogram of myeloma bound was calculated as shown in Table I.

As seen in the final column, the binding efficiencies, expressed as percent anti-isotype counts per minute bound per nanogram of bound myeloma varied between 4.6 and 9.4. The fact that these binding efficiencies did not vary by more than a factor of two enabled us to compare binding titers from one subclass to another.

Results

Isotypic Distribution of Anti-TNP Antibody in nu/nu and nu/+ Mice after Injection with TNP-Ficoll or TNP-Levan. To determine whether the presence of a thymus and hence mature T lymphocytes had any influence on the relative amounts of antibody of the IgM, IgG1, IgG2a, IgG2b, and IgG3 isotypes produced after immunization of mice with a TI antigen, congenitally athymic C57BL10/ScN *nu/nu* and thymus-bearing

TABLE I

Comparison of Binding Efficiencies of Various ^{3}H-Labeled Anti-Isotype Antibodies

^{3}H-labeled myeloma protein added to Rακwell (30 ng)		^{3}H myeloma bound	^{3}H-labeled anti-isotype antibody added		Percent ^{3}H-labeled anti-isotype antibody bound	Calculated percent anti-isotype antibody bound per nanograms of myeloma bound
Designation	Isotype		Specificity	Counts per minute		
		ng				
TEPC-183	IgM,κ	0.72	GαIgM	66,263	4.6	6.4
MOPC-21	IgG1,κ	1.26	GαIgG1	56,500	11.8	9.4
CBPC-101	IgG2a,κ	1.23	GαIgG2a	39,482	5.7	4.6
MOPC-195S	IgG2b,κ	0.87	GαIgG2b	63,324	4.4	5.1
FLOPC-21	IgG3,κ	0.72	RαIgG3	70,832	4.6	6.4

nu/+ age- and sex-matched mice were injected with 10 μg TNP-Ficoll or 10 μg TNP-Levan intraperitoneally. Sera from mice taken on days 5.and 10 after immunization were tested for their titers of anti-TNP antibody of the various Ig isotypes. The data in Table II show that both *nu/nu* and *nu/+* mice gave vigorous responses to the two TI antigens. In both sets of mice, the IgM titer was highest at day 5, whereas the IgG titers were highest at day 10. Although the IgM, IgG3, and IgG1 responses in both *nu/nu* and *nu/+* mice were comparable, the IgG2a response was substantially higher (30- to 100-fold) in thymus-bearing *nu/+* mice than in *nu/nu* mice. The IgG2b response was also somewhat higher in *nu/+* mice but this difference was not as pronounced as in the IgG2a response. The results suggest that the congenital absence of a thymus can selectively impair the IgG2a and IgG2b responses to TI-2 antigens.

Comparison of Isotypes of Anti-TNP Antibody Produced by Purified nu/nu and nu/+ B Cells Placed in Identical Environments. The reduced IgG2a and IgG2b responses to the TI antigens TNP-Ficoll and TNP-Levan in *nu/nu* mice vs. *nu/+* mice could be due to the acute absence of T cells at the time the B lymphocytes are triggered by antigen. An alternative explanation is that B cells in *nu/nu* mice are inherently different from *nu/+* B cells because of their retarded or altered maturation in the absence of T lymphocytes.

To test for the latter possibility, we assayed the TNP-Ficoll-induced response of purified *nu/nu* and *nu/+* B lymphocytes that had been transferred into genetically unresponsive hosts (19). 2.5×10^7 anti-Thy-1 + C-treated, Ig^+, C57BL/10 *nu/nu* or *nu/+* spleen cells were injected intravenously into (CBA/N × C57BL/6)F_1 male recipients. 1 d later the mice were immunized with TNP-Ficoll and bled on days 5, 7, 10, 14, and 21 after immunization. The data in Fig. 1 show the kinetics of the IgM, IgG1, IgG2b, and IgG2a anti-TNP antibody responses in recipients and in (CBA/N × C57BL/6)F_1 male mice that had not received cells. Recipients of *nu/nu* B cells and of *nu/+* B cells had comparable anti-TNP antibody titers within each isotype, including IgG2a and IgG2b. *Nu/nu* B cell recipients had a slightly lower IgG2a serum titer on days 5–10 than *nu/+* B cell recipients, but this difference was less than

TABLE II

Analysis of the Antibody Produced in C57BL/10 nu/nu and nu/+ Mice after Injection with TI Antigens

Antigen	Mice tested‡	Serum assayed	Reciprocal of anti-TNP serum titer*				
			IgM	IgG3	IgG1	IgG2b	IgG2a
TNP-Levan	*nu/nu*	prebleed	3,500	ND§	10	2	2
		day 5	150,000	ND	40	40	2
		day 10	40,000	ND	800	800	20
TNP-Levan	*nu/+*	prebleed	2,200	ND	20	2	2
		day 5	102,000	ND	70	200	200
		day 10	52,000	ND	600	1,600	600
TNP-Ficoll	*nu/nu*	day 5	51,200	300	70	3	2
		day 10	15,000	5,000	2,000	120	20
TNP-Ficoll	*nu/+*	day 5	20,000	1,500	160	80	160
		day 10	12,800	4,096	2,560	500	2,000

* Titer expressed as serum dilution at which 1% of added ^{3}H-labeled anti-isotype counts per minute are bound.

‡ Five to seven mice were tested within each group.

§ ND, not done.

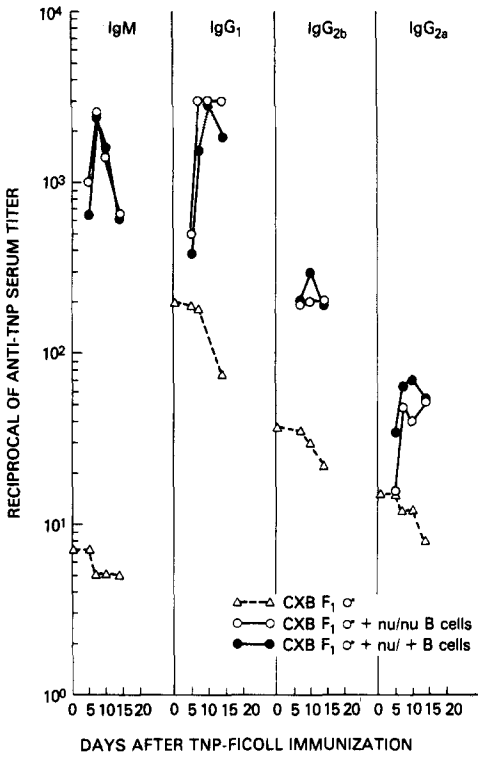

FIG. 1. Comparison of anti-TNP-Ficoll responses of C57BL/10 *nu/nu* and *nu/+* B cells adoptively transferred into genetically unresponsive (CBA/N × C57BL/6)F_1 ♂ recipients. 2.5 × 10^7 anti-Thy-1 + C-treated, Ig^+, spleen cells from *nu/nu* or *nu/+* donors were transferred intravenously into recipients 1 d before injection with TNP-Ficoll. Titers are taken of pools of sera from eight mice within each group.

twofold. As expected, unreconstituted (CBA/N × C57BL/6)F_1 male mice with a defect determined by the X chromosomal gene, *xid* (19), failed to respond to TNP-Ficoll. In fact, the background anti-TNP-titer in these mice dropped as successive bleeds were taken.

Thus, the data from this experiment showed that when *nu/nu* and *nu/+* B cells are transferred into an identical milieu containing T cells, the amounts of the various isotypes of antibody they produce to this TI antigen are comparable.

Effect of T Cell Reconstitution of nu/nu Mice Shortly before Immunization with TNP-Ficoll. The above results suggested that *nu/nu* B cells are inherently as capable of producing IgG2a antibody to a TI antigen as *nu/+* B cells. Thus, the failure of *nu/nu* mice to produce as much IgG2a as *nu/+* mice when stimulated by a TI antigen may reflect an influence of ancillary T cell signals during the immune response. Alternatively, something other than the absence of T cells in the *nu/nu* environment may impair the triggering and/or expansion of IgG2a and IgG2b B cell clones. In an attempt to distinguish between these possibilities, *nu/nu* mice were given 10^7 C57BL T cells 1 d before immunization with TNP-Ficoll. The serum anti-TNP titer of the various isotypes in *nu/nu* mice, T-cell reconstituted *nu/nu* mice, and *nu/+* mice was assayed on days 5–52 after immunization. The data in Fig. 2 show the effect of T cell reconstitution on the isotypes of anti-TNP antibody produced in *nu/nu* mice. Both

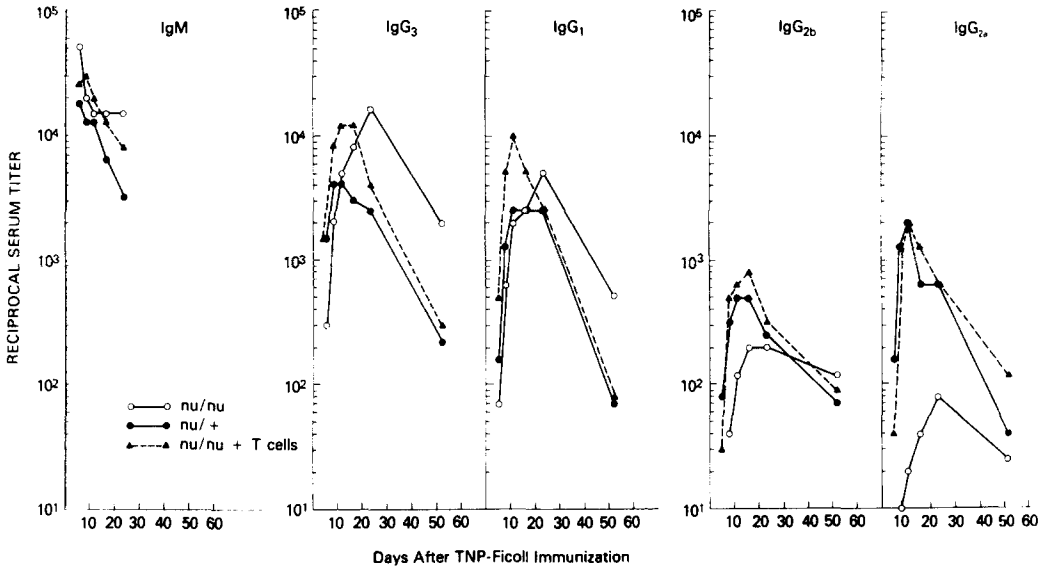

FIG. 2. Effect of transferred syngeneic T cells on isotypes produced by C57BL *nu/nu* mice after TNP-Ficoll immunization. *Nu/nu*, *nu/+*, and *nu/nu* mice reconstituted with 10^7 C57BL splenic T cells 1 d before TNP-Ficoll immunization were compared in their serum anti-TNP titers at various times after immunization with 10 μg TNP-Ficoll. Titers are taken from a pool of six mice per group.

nu/+ and T cell reconstituted *nu/nu* mice showed a dramatic elevation in IgG2a anti-TNP titers when compared with *nu/nu* mice over the entire course of the response. The elevation of IgG2b titers in T cell-bearing mice was substantial but not as pronounced. In contrast, no major effect of T cells on the peak titers of IgM, IgG3, and IgG1 antibody was seen. In both *nu/nu* and T cell-bearing mice, the peak IgM response was found to precede the peak IgG response by several days. In T cell-bearing mice, the peak response of the IgG1, IgG2a, IgG2b, and IgG3 isotypes was seen somewhat earlier than in *nu/nu* mice. In addition, a finding in this and other experiments was that anti-TNP serum titers in T cell-bearing mice generally tended to decline more rapidly than in *nu/nu* mice.

In summary, the two experiments above suggest that the relatively lower IgG2a response made by *nu/nu* mice to the TI antigen TNP-Ficoll is the result of the absence of T lymphocytes during the B cell response rather than the result of a maturational arrest of *nu/nu* B cells or a suppressive influence present in *nu/nu* mice.

To determine the minimal number of T lymphocytes that can affect the *nu/nu* IgG2a response to TNP-Ficoll, graded numbers of T lymphocyte-enriched spleen cells were injected into *nu/nu* mice, and the IgG1 and IgG2a titers were compared on day 16 after TNP-Ficoll immunization. The data in Fig. 3 show that as few as 1.25×10^6 T lymphocytes can cause a significant boost in the IgG2a response of *nu/nu* recipients to TNP-Ficoll. Increasing the number of transferred T cells did not appear to further increase the response. Although 2.5×10^6–10^7 T cells somewhat elevated the IgG1 response in *nu/nu* mice, this increase was less than threefold.

In Table III we have compiled the results from several additional experiments in which the TNP-Ficoll-induced responses of *nu/nu* and T cell-reconstituted *nu/nu* mice were compared. In these experiments, IgG3, IgG1, IgG2b, and IgG2a anti-TNP

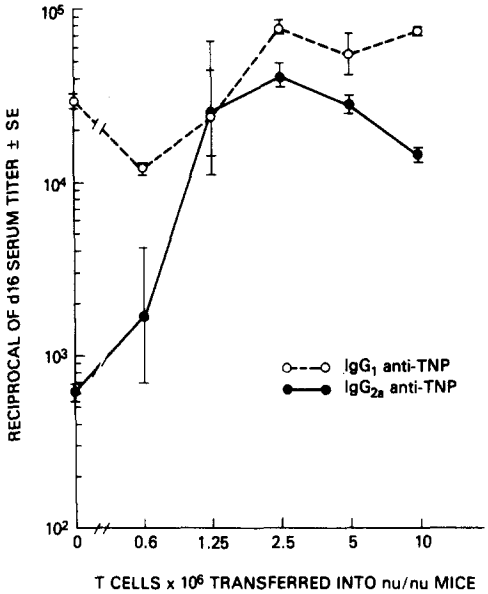

FIG. 3. Effect of number of transferred T cells on the *nu/nu* IgG1 and IgG2a anti-TNP responses to TNP-Ficoll. *Nu/nu* mice were injected with no T cells or 0.6–10 × 10^6 syngeneic splenic T cells 1 d before TNP-Ficoll injection. Results are expressed as the geometric mean of the day-16 serum titers with the relative SE of the mean. Each group contained three to six mice.

TABLE III

Comparison of IgG Subclass Anti-TNP Response in TNP-Ficoll Immunized C57BL/10 nu/nu and T Cell-reconstituted C57BL/10 Mice

Experiment No.	Mice tested	Reciprocal anti-TNP titers in serum 14–20 d after TNP-Ficoll immunization			
		IgG3	IgG1	IgG2b	IgG2a
1	*nu/nu*	8,200	2,500	200	30
	nu/nu + 10^7 C57BL/Ka T cells	12,000	5,100	800	1,280
2	*nu/nu*	32,000	12,800	2,500	20
	nu/nu + 2.5 × 10^7 C57BL/10 T cells	50,000	10,000	20,000	4,000
3	*nu/nu*	42,000	28,000	5,120	500
	nu/nu + 2.5 × 10^6 C57BL/Ka T cells	30,000	36,000	12,000	4,000
4	*nu/nu*	25,600	12,800	1,800	200
	nu/nu + 2.5 × 10^6 C57BL/Ka T cells	12,000	8,000	2,500	2,280

antibody titers were determined on sera taken 14–16 d after immunization. These data reinforce the observation that IgG3 and IgG1 responses to TNP-Ficoll are relatively unaffected by T lymphocytes, whereas the IgG2a and IgG2b responses are positively influenced by the presence of T cells. In every case IgG2a responses are more dramatically affected than IgG2b responses.

In most experiments C57BL/KaLwN T cells were transferred into C57BL10/ScN *nu/nu* mice. Because C57BL/KaLwN and C57BL/10 sublines have some minor differences, the possibility existed that this selective enhancement of IgG2 antibody was the result of a minor allogeneic effect. That this is not the case is demonstrated in experiment 2 in which the transfer of C57BL10/ScN T cells has the same effect.

A trend that becomes obvious when comparing the titers of the various isotypes in the *nu/nu* reponse to TNP-Ficoll is that the strength of the response of the gamma isotypes is in the following order: IgG3 > IgG1 > IgG2b > IgG2a. The presence of T cells, however, shifts this isotypic distribution so that higher levels of IgG2b and IgG2a are produced and this hierarchy in response becomes less pronounced. The validity of comparing the radioimmunoassay titers of the diverse isotypes has been discussed in Materials and Methods.

Discussion

It is now clear that certain antigens, designated TI antigens, can stimulate B cells to proliferate and to differentiate into antibody-producing cells in the absence of detectable ancillary T cell signals (1, 2). This study has shown that B cells in athymic nude mice can be triggered by TNP-Ficoll and TNP-Levan to secrete not only IgM but also IgG1, IgG2a, IgG2b, and IgG3 antibody. Thus B cells can, in an environment containing few, if any, thymus-matured T lymphocytes, produce antibody of many isotypes. Interestingly, the relative amount of anti-TNP antibody of the various isotypic classes produced by nude mice immunized with TNP-Ficoll was found to correlate directly with the 5′ to 3′ heavy chain constant region gene order in the mouse (20). Thus, in a series of experiments, we found that the peak titers of anti-TNP antibodies were in the following order: IgM > IgG3 > IgG1 > IgG2b > IgG2a. It is unlikely that differential catabolism of the various Ig isotypes is responsible for this heirarchy in serum titers. Comparisons of the known catabolic rates of IgM, IgG1, IgG2b, and IgG2a (140, 17, 25, and 13% per day, respectively) (21) with the maximal anti-TNP titers of each isotype measured fail to show any correlations. This observation raises the provocative possibility that the frequency of responding cells of a given isotype is dependent on the distance of the particular constant region gene from the site(s) 5′ to the μ gene reported to be involved in gene switching (22).[2] Whether B cell responses to TI-2 antigens other than TNP-Ficoll and TNP-Levan will also reflect this gene order, and whether it is a feature of all inbred strains, is currently under investigation.

Our predominant interest has been to determine whether these levels of antibody of the various isotypes might be influenced by T lymphocytes and, if such T cell regulation exists, what mechanisms might be involved. Our data indicate that unprimed T lymphocytes can in fact influence the distribution of isotypes produced to TI antigens of the TI-2 class. A positive T cell influence was seen predominantly on IgG2a, an isotype that was poorly produced in athymic *nu/nu* mice. A slight effect of T cells on IgG2b antibody production was also observed. This selective regulatory effect of T cells on IgG2a and IgG2b antibody production did not increase the levels of these isotypes beyond the levels of IgM, IgG3, and IgG1. This is in agreement with the observation of Slack et al. (23) that the IgG3 and IgG1 plaque-forming cell responses of normal thymus-bearing mice to TNP-Ficoll are significantly greater than the combined IgG2a and IgG2b plaque-forming cell responses.

We have considered two major explanations for the preferential helper T cell effect on IgG2 production. (*a*) In normal animals, T cells may exist that can interact with IgG2a-positive B cells, perhaps by recognizing B cell surface IgG2a. This T cell-B cell

[2] Davis, M., S. Kim, and L. Hood. DNA sequences mediating class switching in alpha immunoglobulin genes. *Science (Wash. D. C.)*. In press.

interaction mediated through B cell surface IgG2a may cause preferential expansion of IgG2a clones. The small amount of T cell-expanded IgG2b production could be explained by cross-reactivity of IgG2a and IgG2b determinants or alternatively by the existence of a small population of IgG2b-specific T_H cells. It should be noted that this explanation for the increased IgG2a responses seen in the presence of T cells requires that the responding B cells have undergone the IgM to IgG switch, and express membrane IgG2a before the action of these T cells. (*b*) T cells might, alternatively, induce greater Ig heavy chain gene switching in virgin B cell clones responding to TNP-Ficoll. Thus, additional activation signals given by T cells at the time of B cell triggering may promote the process of gene switching in such a way as to make it more likely that distal constant region genes, such as IgG2a, are expressed. The specificity of T cells that might deliver such signals is not obvious. It is unlikely that they are Ficoll-specific, in that Ficoll is not considered an effective immunogen. DNP hapten-specific helper T cells, on the other hand, have been demonstrated in primed T cell populations (24). Although the effective T cell populations used here have not been primed, we cannot exclude the possibility that they have such specificity for TNP. Finally, the ancillary T cells may have specificity for a membrane-bound B cell determinant, perhaps Lyb3, Lyb5, Lyb7, or Ia (25–27).

We are not yet able to distinguish between these two major mechanisms by which T cells may enhance IgG2a production by B cells. It is clearly of interest to determine the specificity of the effector T cell population and to determine whether it interacts with B cells to result in an increased number of IgG2a-positive clones or simply increased IgG2a production from already differentiated clones.

When investigating the response kinetics of the various isotypes in T cell-bearing and athymic mice, several observations were made with regard to the rapidity and the prolongation of the response. First, in mice bearing T cells, the peak IgG1, IgG3, IgG2b, as well as IgG2a, antibody titers were generally seen earlier than in nude mice. This suggests that T cells may subtly influence the rate of differentiation to IgG production. It should be noted again that, in terms of magnitude of the overall response, only the IgG2a and IgG2b isotypes were affected by T cells. Because IgM production was not assayed prior to day 5 after immunization, a time of peak serum IgM levels, we cannot determine whether T cells accelerated the IgM response. It has recently been shown that the in vitro IgM anti-TNP response of mouse spleen cells to TNP-Ficoll can be markedly diminished by vigorous depletion of T lymphocytes (28). It is conceivable that this effect is related to the capacity of T cells to accelerate B cell antibody production.

In addition to hastening the production of IgG after TNP-Ficoll immunization, the presence of T cells also caused a more pronounced fall in antibody titer after peak production had been achieved. Recent studies (29) have suggested that this might be the result of T cell-dependent anti-idiotype-induced regulation of the TNP-Ficoll response.

In numerous experiments in which the kinetics of the response of the various isotypes was investigated, peak titers of each isotype were generally seen at the same time. This suggests that in the differentiation of a TNP-Ficoll responsive B cell, sequential switching of a given B cell through IgM → IgG3 → IgG1 → IgG2b → IgG2a stages is unlikely. Instead it suggests that, if an antigen-induced switching event does take place, a more likely used differentiation pathway is one in which

progeny of a given B cell clone each diverge and switch from an IgM-positive stage to an IgG3, IgG1, IgG2b, or IgG2a-positive stage at approximately the same time.

These experiments have suggested that the Ig constant region gene order may inherently control the amount of antibody of the various isotypes produced when B lymphocytes are stimulated by TI-2 antigens. Furthermore, our results suggest that the isotypic distribution is influenced by additional signals given by T lymphocytes. It is possible that, in B cell responses to every type of antigen, the deviation from a heirarchic isotypic response reflecting gene order is dependent on the nature of additional signals that a responding virgin B cell received, i.e., strong or weak T cell signals or strong or weak polyclonal activating signals inherent to the antigen. The delivery of strong signals to B lymphocytes may cause an increase in heavy chain gene switching. Finally, helper T cells that recognize specific isotypic determinants on B cell progeny that have switched their membrane isotype could even further expand the production of certain isotypes over that of others. The combined effects of these various antigenic and cellular signals would then create the isotypic distribution characteristic of a B cell response to a given antigen.

Summary

The effect of T lymphocytes on the IgM, IgG3, IgG1, IgG2b, and IgG2a responses of B lymphocytes to the type-2 T-independent antigens, trinitrophenylated (TNP)-Ficoll, and TNP-Levan, was investigated. T cell-bearing *nu/+* mice were found to produce substantially higher IgG2 serum anti-TNP antibody than their athymic counterparts, with *nu/nu* and *nu/+* IgG2a titers exhibiting more disparity than *nu/nu* and *nu/+* IgG2b titers. The IgM, IgG3, and IgG1 anti-TNP levels in *nu/nu* and *nu/+* mice were indistinguishable. By cell transfer experiments, it was determined that this variance in nude and heterozygote IgG2 responses could not be explained by B cell differences between the two strains or by suppressive effects on IgG2 production within *nu/nu* mice. Rather, the difference was shown to be the result of the absence of T cells at the time B cells were responding to antigen. In the absence of T cells, the strength of the *nu/nu* anti-TNP antibody response was found to be in the following order: IgM > IgG3 > IgG1 > IgG2b > IgG2a, a heirarchy identical with the recently proposed heavy chain gene order. The possibilities that T cells influence IgG2 production via their specific recognition of IgG2-bearing B cells or via signals to increase heavy chain switching of responding B cell clones are discussed.

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References

1. Sharon, R., P. R. B. McMaster, A. M. Kask, J. D. Owens, and W. E. Paul. 1975. DNP-Lys-Ficoll: a T-independent antigen which elicits both IgM and IgG anti-DNP antibody-secreting cells. *J. Immunol.* **114**:1585.
2. Barthold, D. R., B. Prescott, P. W. Stashak, D. F. Amsbaugh, and P. J. Baker. 1974.

Regulation of the antibody response to type III pneumococcal polysaccharide. III. Role of regulatory T cells in the development of an IgG and IgA antibody response. *J. Immunol.* **112:**1042.

3. Bottomly, K., and D. E. Mosier. 1979. Mice whose B cells cannot produce the T15 idiotype also lack an antigen-specific helper T cell required for T15 expression. *J. Exp. Med.* **150:** 1399.
4. Hetzelberger, D., and K. Eichmann. 1978. Recognition of idiotypes in lymphocyte interactions. I. Idiotypic selectivity in the cooperation between T and B lymphocytes. *Eur. J. Immunol.* **8:**846.
5. Woodland, R., and H. Cantor. 1978. Idiotype-specific T helper cells are required to induce idiotype-positive B memory cells to secrete antibody. *Eur. J. Immunol.* **8:**600.
6. Kishimoto, T., and K. Ishizaka. 1973. Regulation of antibody response in vitro. VI. Carrier-specific helper cells for IgG and IgE antibody response. *J. Immunol.* **111:**720.
7. Janeway, Jr., C. A., R. A. Murgita, F. I. Weinbaum, R. Asofsky, and H. Wigzell. 1977. Evidence for an immunoglobulin-dependent antigen-specific helper T cell. *Proc. Natl. Acad. Sci. U. S. A.* **74:**4582.
8. Elson, C. O., J. A. Heck, and W. Strober. 1979. T-cell regulation of murine IgA synthesis. *J. Exp. Med.* **149:**632.
9. Herzenberg, L. A., K. Okumura, H. Cantor, V. L. Sato, F.-W. Shen, E. A. Boyse, and L. A. Herzenberg. 1976. T-cell regulation of antibody responses: demonstration of allotype-specific helper T cells and their specific removal by suppressor T cells. *J. Exp. Med.* **144:**330.
10. Mosier, D. E., I. M. Zitron, J. J. Mond, A. Ahmed, I. Scher, and W. E. Paul. 1977. Surface immunoglobulin D as a functional receptor for a subclass of B lymphocytes. *Immunol. Rev.* **37:**89.
11. Bona, C., R. Hooghe, P. A. Cazenave, C. Leguérn, and W. E. Paul. 1979. Cellular basis of regulation of expression of idiotype. II. Immunity to anti-MOPC-460 idiotype antibodies increases the level of anti-trinitrophenyl antibodies bearing 460 idiotypes. *J. Exp. Med.* **149:** 815.
12. Roos, D., and J. A. Loos. 1970. Changes in the carbohydrate metabolism of mitogenically stimulated human peripheral lymphocytes. I. Stimulation by phytohaemagglutinin. *Biochim. Biophys. Acta.* **222:**565.
13. von Boehmer, H., and K. Shortman. 1973. The separation of different cell classes from lymphoid organs. IX. A simple and rapid method for removal of damaged cells from lymphoid cell suspensions. *J. Immunol. Methods.* **2:**293.
14. Mage, M. G., L. L. McHugh, and T. L. Rothstein. 1977. Mouse lymphocytes with and without surface immunoglobulin: preparative scale separation in polystyrene tissue culture dishes coated with specifically purified anti-immunoglobulin. *J. Immunol. Methods.* **15:**47.
15. Rittenberg, M. B., and A. A. Amkraut. 1966. Immunogenicity of trinitrophenyl-hemocyanin: production of primary and secondary anti-hapten precipitins. *J. Immunol.* **97:**421.
16. Green, M. C. 1979. Genetic nomenclature for the immunoglobulin loci of the mouse. *Immunogenetics.* **8:**89.
17. Ey, P. L., S. J. Prowse, and C. R. Jenkin. 1978. Isolation of pure IgG_1, IgG_{2a} and IgG_{2b} immunoglobulins from mouse serum using protein A-Sepharose. *Immunochemistry.* **15:**429.
18. Wilder, R. L., C. C. Yuen, B. Subbarao, V. L. Woods, C. B. Alexander, and R. G. Mage. 1979. Tritium (^3H) radiolabeling of protein A and antibody to high specific activity: application to cell surface antigen radioimmunoassays. *J. Immunol. Methods.* **28:**255.
19. Scher, I., A. D. Steinberg, A. K. Berning, and W. E. Paul. 1975. X-linked B-lymphocyte immune defect in CBA/N mice. II. Studies of the mechanisms underlying the immune defect. *J. Exp. Med.* **142:**637.
20. Honjo, T., and T. Kataoka. 1978. Organization of immunoglobulin heavy chain genes and allelic deletion model. *Proc. Natl. Acad. Sci. U. S. A.* **75:**2140.

21. Fahey, J. L., and S. Sell. 1965. The immunoglobulins of mice. V. The metabolic (catabolic) properties of five immunoglobulin classes. *J. Exp. Med.* **122:**41.
22. Maki, R., A. Traunecker, H. Sakano, W. Roeder, and S. Tonegawa. 1980. Exon shuffling generates an immunoglobulin heavy chain gene. *Proc. Natl. Acad. Sci. U. S. A.* **77:**2138.
23. Slack, J., G. P. Der-Balian, M. Nahm, and J. M. Davie. 1980. Subclass restriction of murine antibodies. II. The IgG plaque-forming cell response to thymus-independent type 1 and type 2 antigens in normal mice and mice expressing an X-linked immunodeficiency. *J. Exp. Med.* **151:**853.
24. Yamashita, U., and M. Kitagawa. 1974. Induction of anti-hapten antibody response by hapten-isologous carrier conjugate. I. Development of hapten-reactive helper cells by hapten-isologous carrier. *Cell. Immunol.* **14:**182.
25. Huber, B., R. K. Gershon, and H. Cantor. 1977. Identification of a B-cell surface structure involved in antigen-dependent triggering: absence of this structure on B cells from CBA/N mutant mice. *J. Exp.Med.* **145:**10.
26. Subbarao, B., A. Ahmed, W. E. Paul, I. Scher, R. Lieberman, and D. E. Mosier. 1979. Lyb-7, a new B cell alloantigen controlled by genes linked to the IgC_H locus. *J. Immunol.* **122:**2279.
27. Mond, J. J., E. Sehgal, D. H. Sachs, and W. E. Paul. 1979. Expression of Ia antigen on adult and neonatal B lymphocytes responsive to thymus-independent antigens. *J. Immunol.* **123:**1619.
28. Mond, J. J., P. K. A. Mongini, D. Sieckmann, and W. E. Paul. 1980. Role of T lymphocytes in the response to TNP-AECM-Ficoll. *J. Immunol.* **125:**1066.
29. Schrater, A. F., E. A. Goidl, G. J. Thorbecke, and G. W. Siskind. 1979. Production of auto-anti-idiotypic antibody during the normal immune response to TNP-Ficoll. III. Absence in nu/nu mice: evidence for T-cell dependence of the anti-idiotypic-antibody response. *J. Exp. Med.* **150:**808.