

T CELL REGULATION OF IMMUNOGLOBULIN CLASS
EXPRESSION IN THE ANTIBODY RESPONSE
TO TRINITROPHENYL-FICOLL

Evidence for T Cell Enhancement of the Immunoglobulin Class Switch*

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Signals from thymus-processed T cells are important in determining the quantities of various immunoglobulin (Ig) classes of antibody (Ab)¹ synthesized in response to antigen (1–6). We have recently reported that T cells can preferentially enhance the production of certain IgG isotypes, namely IgG_{2a}, in the B cell response to the type 2 T-independent (TI-2) antigen,² trinitrophenyl (TNP)-Ficoll (7). Thymus-processed T cells were not mandatory for IgG synthesis in the response to TNP-Ficoll, since athymic nude mice could mount IgG anti-TNP responses to this antigen, including a weak IgG_{2a} response. Interestingly, the relative amount of Ab in each of the several IgG classes produced by nude mice to TNP-Ficoll was found to correlate with the 5' to 3' gene order for the various IgG heavy chain constant region genes (9, 10), i.e., IgG₃ > IgG₁ > IgG_{2b} > IgG_{2a}. This suggested that the relative proximity of a given IgG heavy chain (*Igh-γ*) gene to the switch sites located 5' to the *Igh-μ* gene (11, 12) may influence the frequency of specific isotype switching events that take place within a TNP-Ficoll-responsive B cell clone. The relative effectiveness of T cells at enhancing Ab secretion within each of the IgG classes appeared to correlate inversely with the relative amount of Ab produced in the absence of T cells, i.e., help for IgG_{2a} synthesis > help for IgG_{2b} synthesis > help for IgG₁ synthesis.

One possible explanation for these results is that T cells provide additional signals to TNP-Ficoll-responsive B cells to enhance switching events within the expanding B cell clones. Thus, isotype-switching events that occur with low frequency in the absence of T cells, e.g., the switch to IgG_{2a}, may be the most affected by ancillary T

* Supported in part by Uniformed Services University grant protocol CO7305 to Eleanor Metcalf.

‡ Supported by a fellowship from the Arthritis Foundation.

¹ Abbreviations used in this paper: Ab, antibody; AECM, aminoethylcarbamyl-methyl; BSA, bovine serum albumin; C, complement; DNP, dinitrophenyl; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; R, rabbit; RIA, radioimmunoassay; RT, room temperature; TI-2, type 2 T-independent antigen; TNP, trinitrophenyl.

² In our nomenclature a TI-2 antigen denotes an antigen that can stimulate an *in vivo* Ab response in the absence of thymus-matured T cells (i.e., in *nu/nu* mice) but that fails to induce such a response in mice with the *xid*-determined defect (7). In *in vitro* culture, Thy-1⁺, Lyt-1⁺ cells have been shown to be necessary for Ab responses to TI-2 antigens (8). To avoid confusion concerning the role of T cells in these responses, we suggest that the designation "type 2 antigen" be used.

cell signals. Alternatively, the preferential T cell enhancement of IgG_{2a} synthesis may be a result of IgG_{2a} isotype-specific T cells. These T cells may not affect the entire TNP-Ficoll-responsive clone per se, but rather, may selectively interact only with membrane IgG_{2a}-positive cells generated during the response. This interaction with IgG_{2a}-positive daughter cells could conceivably cause an expansion in their number or perhaps in their rate of Ab secretion.

In this report we substantiate the hierarchical order of IgG class expression in responses of nude mice to TNP-Ficoll, confirm the preferential effect of T cells at enhancing TNP-Ficoll-induced IgG_{2a} production, and provide evidence that supports the hypothesis that T cells manifest this effect by providing accessory signals that increase switching events within TNP-Ficoll-responsive B cell clones.

Materials and Methods

Animals. C57BL/10 ScN *nu/nu*, C57BL/10 ScN *nu/+*, C57BL/10 ScN *+/+* mice, and C57BL/KaLwN *+/+* were obtained from the Small Animal Section, Division of Research Services, National Institutes of Health, Bethesda, MD. Mice were generally used at 2-3 mo of age.

Antigens. Trinitrophenyl-aminoethylcarbamylmethyl (TNP-AECM)-Ficoll, which will be abbreviated TNP-Ficoll, (mol wt = 400,000; 55 mol TNP/mol Ficoll) was purchased from Biosearch (San Rafael, CA). This was used in all experiments other than the splenic focus assay. TNP-AECM-Ficoll (28 mol TNP/mol Ficoll), a gift of Dr. Alfred Singer, National Cancer Institute, National Institutes of Health, was used in the latter assay. Trinitrophenylated-bovine serum albumin (TNP-BSA) at conjugation ratios of 5.1 mol TNP/mol BSA and 10.2 mol TNP/mol BSA were prepared by a modification of the method of Rittenberg and Amkraut (13), in which various concentrations of trinitrobenzenesulfonic acid (Pierce Chemical Co., Rockford, IL) were reacted with a given amount of BSA.

Selective Enrichment for B or T Cell Preparations. To enrich selectively for B cells for use in the splenic focus assay (described below), *nu/+* spleen cell populations were processed in the following manner. Erythrocytes, dead cells, and Thy-1.2-positive cells were removed by procedures previously described (7). The cells were then treated with a monoclonal arsanilate-conjugated anti-Lyt-1 reagent (Becton, Dickinson & Co., Sunnyvale, CA) at a concentration of 10 μ g purified Ab/ $3-4 \times 10^6$ cells per ml for 30 min at room temperature (RT), centrifuged, and incubated with rabbit (R) anti-arsanilate (Becton, Dickinson & Co.) at a concentration of 16-20 μ g purified immunoglobulin/ $3-4 \times 10^6$ cells per ml for an additional 30 min at RT. The cells were again centrifuged and finally incubated in a 1:20 dilution of preselected R complement (C) at a cell concentration of $3-4 \times 10^6$ cells/ml at 37°C for 45 min. The final viable lymphocyte yield after the anti-Thy-1.2 and anti-Lyt-1 cytotoxic procedures was ~30% of the starting population.

To enrich selectively for T cells, spleen cell suspensions were processed by the method described by Mage et al. (14), which has been used previously by this laboratory (7). T cell preparations to be used in the splenic focus assay were taken through an additional step to remove potential contaminating B cells, i.e., they were treated with a 1:2 dilution of culture supernatant from a hybridoma-secreting anti-ThB. This reagent recognizes a determinant present on most B cells but not on mature T cells (15) and was kindly donated by Dr. John Kung, National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIAID, NIH). Cells were incubated with anti-ThB at a density of $3-4 \times 10^6$ cells/ml for 30 min at RT and then with a 1:20 dilution of R C at 37°C for 45 min. The viable lymphocyte yield after goat anti-Ig-coated plate treatments (twice) and an anti-ThB and C treatment was typically 15-18% of the starting population.

Radioimmunoassay (RIA) for Measurement of Soluble Anti-TNP Antibody. The solid-phase RIA described previously (7), which employs TNP-BSA-coated flexible microtiter plates and ³H-labeled anti-isotope reagents, was used to measure anti-TNP antibody of the various classes found in immune serum and in splenic focus assay culture supernatants.

Cell Culture RIA for Secretion of Anti-TNP Antibody from Short-term Cultured Cells. A cell culture

RIA for the detection of antibody produced by secretory cells has been described by us elsewhere (16). Briefly, cells are cultured on TNP-BSA-precoated flexible flat-bottomed microtiter wells for 24 h at 37°C in a humidified 5% CO₂ atmosphere. Various numbers of immune cells were always tested to insure that a dose was obtained in which secreted antibody did not saturate the binding capacity of the antigen-coated wells. After 24 h of culture, the cells were washed away and the amount of bound anti-TNP Ab was measured with the use of ³H-labeled anti-isotype reagents.

³H-labeled Anti-Isotype Reagents. The production of the various classes of anti-TNP Ab was quantitated with the use of affinity-purified anti-isotype reagents (anti-IgM, anti-IgG₁, anti-IgG_{2a}, anti-IgG_{2b}, and anti-IgG₃) which had been tritiated with NaB₃H₄ as described previously (7, 17). These reagents have been shown to be specific and to have comparable binding efficiencies for their respective insolubilized Ig class substrates (7). Because of their comparable binding efficiencies, we have considered it legitimate to compare binding titers (in our serum analyses) from one isotype to another, and to compare percentage-bound values (in our in vitro secretion analyses) from one isotype to another.

In our RIA for soluble Ab, the [³H]anti-IgG₁ and the [³H]anti-IgG_{2b} reagents are both capable of detecting a minimum of ~0.2 ng/ml of purified monoclonal anti-TNP Ab of the IgG₁ and IgG_{2b} isotypes, respectively (16). Because all the anti-isotype reagents have comparable binding efficiencies, the sensitivity of the RIA for detection of anti-TNP Ab of other classes when the anti-IgM, anti-IgG₃, and anti-IgG_{2a} reagents are used is likely also to be ~0.2 ng/ml.

Assay for Relative Affinities of Anti-TNP Antibodies. Herzenberg et al. (18) have recently described a method to assess Ab affinity. In this technique the ability of an Ab to bind wells coated with TNP-BSA of a relatively low degree of conjugation is compared with its ability to bind to wells coated with TNP-BSA of a higher degree of conjugation. We compared the affinities of various monoclonal anti-TNP reagents and anti-TNP sera by analysis of their TNP_{5.1}-BSA/TNP_{10.2}-BSA binding ratios. Briefly, the microtiter wells were coated for 1 h with 50 μl of TNP_{5.1}-BSA or TNP_{10.2}-BSA in saline at a concentration of 100 μg/ml, washed, and incubated with 50 μl of various dilutions of anti-TNP Ab in phosphate-buffered saline, pH 7.4 (PBS) + 1% BSA + 0.1% sodium azide + 0.005 M disodium ethylenediamine tetraacetate (assay buffer) for ~1-2 h. The wells were then washed, and 50 μl (~30,000 cpm) of the appropriate ³H-labeled anti-isotype reagent in assay buffer was added to each well. After ~2 h of incubation, the wells were washed again and the amount of anti-TNP Ab bound specifically to the wells was analyzed by measuring the counts per minute of the ³H-labeled anti-isotype reagent bound. Binding curves were established by plotting the log of the counts per minute bound by the log of anti-TNP Ab dilution tested. The ratio of TNP_{5.1}-BSA/TNP_{10.2}-BSA binding was obtained by comparing the counts per minute bound at a given dilution within the linear region of the binding curves.

The purified MOPC-315 and MOPC-460 IgA myeloma proteins used in these assays as monoclonal standards were kindly provided by Dr. Abdulla Rifai (University of Washington Medical School, Seattle, WA) and Dr. Constantin Bona (Mount Sinai School of Medicine, New York), respectively. The monoclonal anti-dinitrophenyl (DNP) hybridoma Ab designated as 3-12-D (IgG₁) and 6-16-A (IgG₁) were generated by M. Scott and Dr. J. Fleischman (Washington University Medical School, St. Louis, MO) were previously studied (17) and were provided to us by Dr. Leonore Herzenberg (Stanford University Medical School, Stanford, CA).

The reported affinities of these various reagents are MOPC-315, $K_a = 2 \times 10^7$ L/M for TNP (19); MOPC-460, $K_i = 1.2 \times 10^6$ L/M for TNP (20); 3-12-D, $K_a = 1.0 \times 10^6$ L/M for DNP (18); and 6-16-A, $K_a = 1.1 \times 10^8$ L/M for DNP (18). In a comparison of the relative binding of the 3-12-D and 6-16-A reagents to wells coated with TNP-BSA vs. DNP-BSA, the 3-12-D hybridoma protein exhibited a much higher affinity for DNP than for TNP. The 6-16-A reagent, however, bound DNP and TNP to a comparable degree (P. Mongini, unpublished data).

Anti-IgM Treatment of Neonatal Mice. Anti-IgM to be used for this procedure was prepared in the following manner. Goat anti-Mc471B (anti-IgM, κ BALB/c myeloma protein, a kind gift of Dr. Richard Asofsky, NIAID, NIH) was passed over CBPC-101- (IgG_{2a}, κ) and MOPC-195S- (IgG_{2b}, κ) Sepharose affinity columns to remove anti-Fab and anti-IgG activity from the

immune serum. The nonbound serum fractions were then precipitated three times with 50% saturated $(\text{NH}_4)_2\text{SO}_4$ (vol:vol) at 4°C, dialyzed in PBS, centrifuged at 15,000 rpm for 30 min to remove aggregates, and sterilely filtered. A rough analysis of Ab activity was performed by comparing the ability of various concentrations of this preparation and of affinity-purified anti-IgM protein derived from this preparation (prepared by Dr. Anthony DeFranco, NIAID, NIH) at precipitating TEPC-183 in gel diffusion. 0.5 mg/ml of the $(\text{NH}_4)_2\text{SO}_4$ -precipitated preparation gave as strong a precipitin line as 0.125 mg/ml affinity-purified goat anti-IgM. Thus, we can estimate that approximately one-fourth of the protein in this preparation is anti-IgM.

For anti-IgM treatment, neonatal C57BL/10 mice were injected intraperitoneally with 0.1 ml (1.6 mg) of the above preparation within 12 h of birth. This treatment was continued daily for the first week; every other day thereafter the mice were given 0.15 ml (2.4 mg) intraperitoneally until they were killed at 2 mo of age. When they had been killed the mice were found, by gel-diffusion analysis, to have anti-IgM in their serum and to have no detectable serum levels of IgM. The spleens of these mice were found to have 3% Ig^+ cells, whereas untreated age-matched C57BL/10 mice had 30% Ig^+ cells in their spleens as determined by fluorescence microscopy.

Splenic Focus Assay for Enumeration of Anti-TNP-Ficoll-responsive Clones. A modification of the splenic focus assay described previously (21, 22) was used to analyze the isotypes produced by TNP-Ficoll-responsive B cell clones. Briefly, limiting numbers of unprimed B cells with or without a given number of unprimed T cells were transferred intravenously into lethally irradiated unprimed recipients. To insure the complete absence of thymus-matured T cells, the recipients used were 1,600-rad ^{137}Cs -irradiated C57BL/10 ScN *nu/nu* mice. Approximately 15 h after cell transfer, the recipients were injected with 10 μg TNP-AECM-Ficoll intravenously and after 1 h were killed, and splenic fragments prepared and placed into culture. Culture supernatants were changed every 3–4 d. Supernatants from days 7 through 17 were tested for anti-TNP antibody of the various isotypes by the RIA described above.

A preliminary analysis of the relationship of B cell dose to number of positive fragments was consistent with our data following a Poisson distribution (23). We have used a dose of $2.5\text{--}5 \times 10^6$ B cells per recipient. This dose allowed us to maintain the frequency of total positive fragments <27% and yet to measure the frequency of very infrequently produced Ab isotypes, e.g., IgG_{2a} , without expanding the number of fragments tested to an unmanageable size.

In the splenic focus assay we were concerned with the positivity or negativity of a given fragment for the production of a given isotype of anti-TNP Ab. The cutoff in counts per minute anti-isotype bound which was used to categorize supernatants as positive or negative in our RIA was determined in two ways: (a) by calculating the $\bar{X} \pm 3 \text{SD}$ of the counts per minute bound in all splenic fragments from mice given B cells only (no antigen) or B and T cells (no antigen), and (b) by constructing a histogram of the percentage of fragments (from mice which received cells and antigen) that fall into ascending counts per minute-bound categories and determining the point at which a trough in the bimodal distribution of positive and negative fragments appeared (24). In general, the cutoff point for positivity was the same with both of these approaches.

Results

Preferential T Cell Enhancement of IgG_{2a} Ab Response to TNP-Ficoll. The data in Table I demonstrate our recently described observation that T cells can exert what appears to be an IgG subclass-specific effect on the antibody response to the TI-2 antigen, TNP-Ficoll. When the anti-TNP-Ficoll serum responses of *nu/nu* and syngeneic T cell reconstituted *nu/nu* mice were compared, it was found that T cells caused a preferential enhancement of the IgG_{2a} and IgG_{2b} responses; the IgG_{2a} response was increased to a more significant degree than the IgG_{2b} response. However, the IgG_1 , IgG_3 , and IgM responses were not substantially different between the two groups of mice. In addition, this experiment illustrates the highly reproducible isotype hierarchy seen when the quantities of serum Ab of the various classes produced in nonreconstituted nude mice are compared, i.e., $\text{IgM} > \text{IgG}_3 > \text{IgG}_1 > \text{IgG}_{2b} > \text{IgG}_{2a}$.

TABLE I
Analysis of Ab Produced in *nu/nu* and in T cell-reconstituted *nu/nu* Mice After Immunization with the Type 2 Antigen TNP-Ficoll

Mice tested*	Reciprocal of peak anti-TNP serum titer‡				
	IgM	IgG ₃	IgG ₁	IgG _{2b}	IgG _{2a}
<i>nu/nu</i>	51,200	16,380	5,120	200	80
<i>nu/nu</i> + T cells	30,000	12,000	10,240	800	2,000

* Five mice tested within each group; 10^7 normal C57BL/Ka T cells were injected intravenously into C57BL/10 *nu/nu* recipients 1 d before intraperitoneal immunization with 10 μ g TNP-Ficoll in saline. Sera collected at days 5, 7, 10, 14, and 20 after immunization were tested for anti-TNP Ab of the various isotypes.

‡ Titer expressed as serum dilution at which 1% of added 3 H-anti-isotype counts per minute are bound.

TABLE II
Comparison of In Vitro 24-h Secretion of Anti-TNP Antibody by Spleen Cells from TNP-Ficoll (TF)-immunized *nu/nu* vs. *nu/nu* Plus T Cell-primed Mice*

Spleen cells tested	Percentage of anti-isotype bound/ 2.5×10^5 spleen lymphocytes				
	IgM	IgG ₃	IgG ₁	IgG _{2b}	IgG _{2a}
			$\bar{X} \pm SE$		
Nonimmune <i>nu/nu</i>	1.23	0.01	0.01	0.01	0.05
TF-immune <i>nu/nu</i>	50.41 \pm 2.71	13.52 \pm 4.71	5.25 \pm 0.06	1.39 \pm 0.51	0.65 \pm 0.19
TF-immune <i>nu/nu</i> + T cells	56.11 \pm 0.31	24.69 \pm 7.40	10.46 \pm 0.65	5.01 \pm 0.90	3.87 \pm 0.88
Ratio of <i>nu/nu</i> + T cells/ <i>nu/nu</i>	1.11	1.83	1.99	3.60	5.95

* 2.5×10^5 immune or nonimmune spleen lymphocytes were incubated in TNP-BSA-coated flexible microtiter wells for 24 h at 37°C. The cells were then washed away and the amount of each isotype bound to the wells quantitated by the addition of $\sim 30,000$ cpm of 3 H-anti-IgM, 3 H-anti-IgG₃, 3 H-anti-IgG₁, 3 H-anti-IgG_{2b}, and 3 H-anti-IgG_{2a} to the appropriate wells. Spleen cells from groups of mice immunized 5, 7, 9, 11, and 13 d previously were tested. The data shown in the peak response of each class of Ab. Each value is the $\bar{X} \pm SE$ of spleen cells from three individual mice.

To exclude the possibility that this isotypic hierarchy resulted from differing rates of in vivo immunoglobulin clearance, we have compared the in vitro secretion of Ab from spleen cells of immune *nu/nu* or T cell-reconstituted *nu/nu* mice. Table II presents data from an experiment in which the in vitro secretion of Ab from spleen cells of mice immunized 5, 7, 9, 11, and 13 d previously with TNP-Ficoll was analyzed. The results shown are the peak amounts of anti-TNP Ab of each isotype produced. The results confirm the isotypic hierarchy observed when serum Ab levels were analyzed and eliminate the possibility that the differences in the serum levels of various classes of anti-TNP-Ficoll Ab were due to differences in in vivo clearance rates.

In addition, the in vitro spleen cell secretion data presented in Table II confirm the preferential relative enhancement of IgG_{2a} Ab synthesis by T cells. These data indicate that T cells can occasionally cause an enhancement in other IgG subclasses as well. Interestingly, the relative enhancement of each of the isotypes by T cells inversely correlates with the relative synthesis of each of the isotypes in the absence of added T cells. In the numerous experiments in which serum responses have been analyzed, the

TABLE III
Comparison of Affinities of Various Anti-TNP Antibodies by Comparing Differential RIA Binding to TNP₅BSA and TNP_{10.2}BSA*

Antibody tested	TNP ₅ BSA/TNP _{10.2} BSA binding ratio					Affinity for TNP $K_a (M^{-1})$
	Experiment number					
	1	2	3	4	5	
<i>nu/nu</i> IgG _{2a} anti-TF	0.58 ± 0.10	0.47	0.68	0.59	—	—
<i>nu/nu</i> + T cells IgG _{2a} anti-TF	0.49 ± 0.04	0.62	0.67	0.58	—	—
MOPC-460	0.74	0.76	—	—	0.83	1.2 × 10 ⁵
MOPC-315	1.30	1.28	—	—	1.03	2 × 10 ⁷
3-12-D	0.17	—	—	—	—	≪ 1.0 × 10 ⁶ ‡
6-16-A	1.00	—	—	—	—	~ 1.1 × 10 ⁸ ‡

* This RIA was performed and the binding ratios calculated as described in Materials and Methods. In Exp. 1, the binding ratios of individual sera from a group of TF-immune mice were analyzed; results are shown as $\bar{X} \pm SE$. In the remaining experiments serum pools from immune mice were tested.

‡ The K_a of 3-12-D and 6-16-A for TNP were not available; however, the K_a of 3-12-D and 6-16-A for DNP have been reported to be 1.0×10^6 and 1.1×10^8 , respectively. As a result of comparing the binding of these monoclonal proteins to DNP₄₂-BSA and TNP₁₅-BSA we concluded that 3-12-D binds TNP with much less affinity than DNP and that 6-16-A has approximately the same binding affinity for TNP and DNP.

enhancement of IgG₃ and IgG₁ responses by T cells has been occasionally observed but is not a reproducible phenomenon. The enhancement of the IgG₂ responses, most notably the IgG_{2a} response, has been consistently observed.

Comparison of the Affinities of IgG_{2a} Anti-TNP-Ficoll Ab in nu/nu and in T Cell-reconstituted nu/nu Mice. We wished to exclude the possibility that the preferential T cell effect on IgG_{2a} Ab synthesis might be a result of changes in the IgG_{2a} antibody's affinity for antigen rather than in the antibody's concentration. This was a potential concern because high affinity Ab might be more easily detected in the RIA we employed. Therefore, we compared the relative affinities of IgG_{2a} anti-TNP-Ficoll Ab produced by *nu/nu* and T cell-reconstituted *nu/nu* mice by assessing their differential binding to wells coated with TNP-BSA of varying degrees of conjugation (18). The data in Table III show that in this assay, which is capable of distinguishing a range of equilibrium constants from 10⁵ L/M to >10⁷ L/M, no significant difference in the TNP₅-BSA/TNP_{10.2}-BSA binding ratio of IgG_{2a} Ab from *nu/nu* or T cell-reconstituted *nu/nu* mice could be discerned. Thus, we have found no indication that T cells change the affinity of the IgG_{2a} Ab synthesized to TNP-Ficoll. We are, therefore, reasonably certain that the preferential increases in IgG_{2a} anti-TNP Ab detected by our RIA are a result of increases in IgG_{2a} synthesis.

Ability of T Cells to Enhance the Frequency of TNP-Ficoll-responsive B Cell Clones that Secrete IgG_{2a}. How does the T cell preferentially enhance IgG_{2a} synthesis in the B cell response to the TI-2 antigen TNP-Ficoll? We have previously proposed two possible explanations for this phenomenon: (a) IgG_{2a} isotype-specific T cells may specifically interact with B cells which bear membrane IgG_{2a} and cause their selective expansion or increase in Ab secretion, or (b) T cells of an as yet undefined specificity may affect the activation or expansion of TNP-Ficoll-responsive B cell clones and thereby increase the frequency with which these clones switch to IgG_{2a}.

TABLE IV
Effect of T Cells on the Number of B Cell-containing Fragments Secreting IgG_{2a} Antibody

Cells transferred	TNP-Ficoll	Total number of fragments studied	Total number B cells injected	Number of fragments secreting anti-TNP antibody per 10 ⁶ B cells injected				
				IgM	IgG ₃	IgG ₁	IgG _{2b}	IgG _{2a}
			<i>x 10⁶</i>			$\bar{X} \pm SE$		
B	-	97	12.5	0.05 ± 0.03	0.03 ± 0.03	0	0	0
B + T cells	-	82	5	0.20 ± 0.20	0.20 ± 0.20	0	0	0
B	+	496	40	3.28 ± 0.36	1.68 ± 0.24	0.55 ± 0.07	0.48 ± 0.14	0.02 ± 0.02
B + T cells	+	427	32.5	3.66 ± 0.70	2.52 ± 0.40	0.88 ± 0.23	1.18 ± 0.31	0.46 ± 0.11
<i>P</i>				NS‡	NS	NS	0.05 > <i>P</i> > 0.02	<i>P</i> < 0.001

* Irradiated recipients that received no cells had no positive fragments for any of the isotypes tested; irradiated recipients that received T cells only plus TNP-Ficoll had 0.04 ± 0.03 IgM-positive fragments, 0.01 ± 0.01 IgG₃-positive fragments, 0.04 ± 0.03 IgG₁-positive fragments, and no IgG_{2b} or IgG_{2a} fragments per 10⁶ T cells injected.

‡ Not significant.

In an attempt to distinguish between these two possibilities, we have analyzed the effect of T cells upon the TNP-Ficoll response of individual B cell clones. Through the use of a modification of the splenic focus assay (21, 22), we have compared the isotypes of Ab produced by individual B cell clones in response to TNP-Ficoll in the absence of thymus-matured T cells and in the presence of defined numbers of T cells. If T cells influence IgG_{2a} production in the intact animal by increasing the likelihood that a TNP-Ficoll-responsive B cell clone will switch into IgG_{2a} production, then a higher frequency of anti-TNP IgG_{2a}-secreting fragments should be observed in the presence of T cells than in their absence. If, on the other hand, T cells, e.g., IgG_{2a} isotype-specific T cells, simply enhance the amount of IgG_{2a} Ab produced in clones that had by a T-independent mechanism switched into IgG_{2a} production, then one might expect to find equivalent numbers of IgG_{2a}-secreting fragments in the presence or absence of T cells; T cell-containing fragments would be expected to have a higher concentration of IgG_{2a}. It should be noted that the above comparison would only be valid if the frequency of TNP-Ficoll-responsive clones that produce IgG_{2a} in the absence of T cells is substantially less than the frequency of all TNP-Ficoll-responsive clones.

The basic protocol used in the TNP-Ficoll splenic focus assay was as follows: 2.5–5 × 10⁶ purified B cells were transferred into 1,600-rad-irradiated *nu/nu* recipients with or without 5 × 10⁶ purified syngeneic T cells. 18 h later the recipients were injected with 10 μg TNP-Ficoll intravenously. 1 h after antigen injection, the spleens were excised, sliced into 1-mm fragments, and cultured individually. The frequency of fragments secreting anti-TNP Ab of the IgM, IgG₃, IgG₁, IgG_{2b}, and IgG_{2a} isotypes after 7–17 d of culture is shown in Table IV. In the absence of T cells, the hierarchy in isotype frequency is again consistent with the 5' to 3' *Igh-C* gene order. Although T cells appeared to have no significant effect on the number of fragments secreting IgM, IgG₃, or IgG₁ anti-TNP Ab, the presence of T cells significantly increased the number of fragments secreting detectable IgG_{2b} and IgG_{2a} Ab. Again, the IgG_{2a} response was most affected by the presence of T cells. Indeed, in the absence of T cells, only 1 IgG_{2a}-positive clone was observed out of a total of 496 fragments analyzed. That T cells enhanced the production of IgG_{2a} from purified syngeneic *nu/+* B cells in the splenic focus assay shows that the enhancement of IgG_{2a} anti-TNP-Ficoll Ab in

TABLE V
Analysis of the Number of Isotypes Produced in Fragments Containing TNP-Ficoll-responsive B Cells

Cells transferred	TNP-Ficoll	Number of anti-TNP-secreting fragments	Percentage of individual fragments secreting the following number of isotypes					\bar{X} number of isotypes per fragment
			1	2	3	4	5	
B	+	117	40.9	40.9	10.4	7.8	0	1.85
B + T	+	113	34.5	32.7	15.9	9.7	7.1	2.22

T cell-reconstituted *nu/nu* mice is not due to a unique ability of *nu/nu* B cells to be affected by T cells.

We cannot be certain that every TNP-Ficoll-responsive precursor was receiving optimum T cell help in this splenic focus assay. When greater numbers of T cells were transferred into irradiated *nu/nu* recipients together with a constant number of B cells, suppressive effects appeared to manifest themselves (data not shown). This is consistent with our previous observation that transfer of $>2.5 \times 10^6$ T cells into nonirradiated *nu/nu* mice failed to increase the IgG_{2a} response to TNP-Ficoll above that seen in recipients of 2.5×10^6 T cells. In fact, the IgG_{2a} response of nudes given $\geq 5 \times 10^6$ T cells was somewhat lower than that of nudes given 2.5×10^6 T cells (7).

It is possible that due to the limit in sensitivity of our RIA we have failed to detect fragments that secreted very low levels of IgG_{2a} anti-TNP Ab. The difference in IgG_{2a} production between fragments containing only B cells and those containing B and T cells may therefore be a quantitative rather than qualitative one. We cannot completely rule this out, but we think that it is unlikely for the following reasons. If one assumes a constant secretion rate of 1,000 molecules/cell/s (21, 25) over the course of 3 d of culture, i.e., the interval since the last change in culture supernatant, then a single IgG_{2a}-positive secretory cell should have released enough Ab into the culture supernatant to achieve a concentration of 0.07 ng/200 μ l of supernatant, a concentration which our assay is able to measure. We should, therefore, be able to detect approximately one actively secreting cell. However, because this concentration is at the borderline of the sensitivity of the RIA, and because cells may differ in their secretion rates from the above calculated value, we may conceivably have missed some clones that contain very few IgG_{2a}-secreting daughter cells.

With the above caveat, the results from the splenic focus assay indicate that T cells present within fragments containing individual TNP-Ficoll-responsive B cell precursors cause an increase in the number of IgG_{2a}-secreting clones rather than simply an increase in the amount of IgG_{2a} secreted by positive clones.

Analysis of the Heterogeneity of Isotype Expression within TNP-Ficoll-responsive B Cell Clones. To achieve a clearer understanding of the switching events that occur within TNP-Ficoll-responsive B cell clones in the presence or absence of T cells, we have analyzed the heterogeneity of isotype expression within the TNP-Ficoll-responsive fragments. This further analysis of the splenic focus assay results is shown in Tables V-VII and in Fig. 1.

Table V shows a comparison of the number of isotypes of anti-TNP Ab found in individual fragments containing B cells only vs. those containing T cells and B cells. In the presence of T cells, a greater percentage of fragments was found which secreted

TABLE VI

Analysis of the Number of Isotypes Produced in Fragments Secreting Given Isotypes of Anti-TNP Antibody

Cells transferred	Fragments secreting the following isotype of anti-TNP Ab	n	Percent of individual fragments secreting the following number of isotypes					\bar{X} number of isotypes per fragment
			1	2	3	4	5	
B	IgM	117	39.3	42.0	10.7	8.0	0	1.79
	IgG ₃	63	0	69.8	15.9	14.3	0	2.45
	IgG ₁	21	4.8	14.3	42.9	38.1	0	3.15
	IgG _{2b}	18	11.1	11.1	27.8	50.0	0	3.16
	IgG _{2a}	1	0	0	0	100	0	4.00
B + T	IgM	105	31.4	33.3	16.7	10.8	7.8	2.26
	IgG ₃	74	5.3	46.7	24.0	13.3	10.7	2.81
	IgG ₁	26	0	7.7	26.9	34.6	30.8	3.89
	IgG _{2b}	33	6.1	3.0	39.4	27.3	24.2	3.61
	IgG _{2a}	13	0	7.7	7.7	23.1	61.5	4.38

three to five isotypes. Of the fragments that contained B cells only, none was found to secrete all five isotypes, whereas 7.1% of the anti-TNP-positive fragments containing T and B cells secreted all five isotypes.

Table VI shows a further subdivision of the data in Table V. The fragments secreting anti-TNP Ab are categorized as those secreting IgM, IgG₃, IgG₁, IgG_{2b}, or IgG_{2a} isotypes. The percentage of these fragments that secrete only the designated isotype or which also secrete one to four other isotypes was evaluated. The data again show that fragments that contained B and T cells exhibited a significant increase in the frequency of TNP-Ficoll responsive clones which synthesized all five classes of anti-TNP Ab. This is largely accounted for by the increase in IgG_{2a} expression. Furthermore, the data show that in terms of complexity of isotype expression, fragments secreting IgG_{2a} tend to secrete most of the other isotypes whereas fragments secreting IgM tend to secrete, on the average, fewer additional isotypes. From a probability analysis of our data, it is possible that the co-expression of the more frequently occurring isotypes (i.e., IgM and IgG₃) may, in a minority of fragments, be a result of overlap of two independently assorting precursors. However, the possibility that co-expression of the less frequently occurring isotypes results from precursor overlap is highly unlikely.

When co-expression of the various isotypes is analyzed, the following trend is observed: fragments secreting isotypes encoded by genes on the 5' end of the *Igh-C* gene cluster (5' *Igh-μ*, *Igh-γ3*, *Igh-γ1*, *Igh-γ2b*, *Igh-γ2a* 3') tend, on the average, to be less heterogeneous in terms of expression of other isotypes, whereas fragments secreting isotypes encoded by genes on the 3' end of the *Igh-C* gene cluster tend to be the most heterogeneous in terms of isotype expression. The data in Fig. 1 and Table VII further illustrate that B and T cell-containing fragments that secrete a given isotype also tend to secrete the isotypes whose *Igh-C* genes are encoded 5' to the given isotype gene. For example, Fig. 1 shows that 92% of fragments secreting IgG₃ also secrete IgM, whereas many fewer secrete isotypes whose *Igh-C* genes are encoded to the 3' end of the *Igh-γ3* gene, i.e., IgG₁, IgG_{2b}, and IgG_{2a}. 96.5% and 88.5% of the IgG₁-positive fragments secrete IgM and IgG₃ respectively, whereas only 69% secrete IgG_{2b} and only 38%

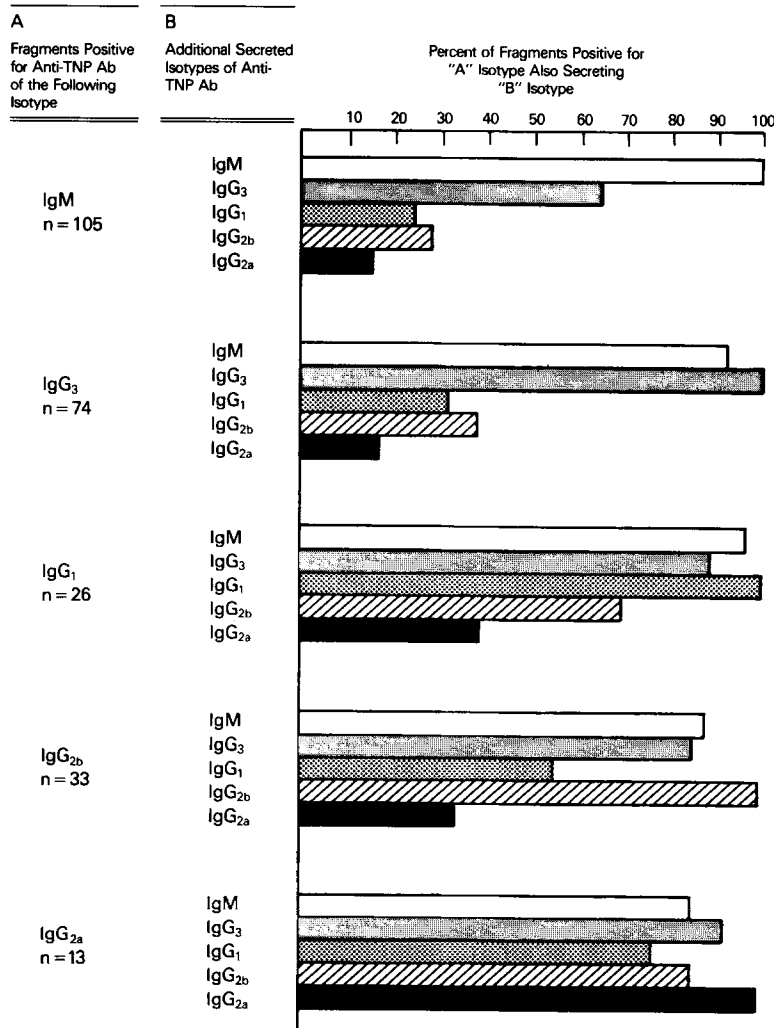


FIG. 1. Analysis of the isotype heterogeneity of the anti-TNP-Ficoll response in fragments containing B + T cells.

secrete IgG_{2a}. Most of the IgG_{2b}-positive fragments secrete IgM and IgG₃; however, only 55% also secrete IgG₁. The failure of almost half of the IgG_{2b}-secreting fragments to secrete detectable IgG₁ is clearly an exception to the trend that fragments secreting a given isotype will also secrete isotypes encoded by 5' *Igh-C* genes. However, in agreement with the noted trend, most of the IgG_{2a}-positive fragments also secrete IgM, IgG₃, IgG₁, and IgG_{2b} Ab.

Whereas Fig. 1 presents the data as the percent of fragments secreting a defined class of Ab which also secretes a given other isotype, Table VII presents an analysis of the frequency of co-expression of all the 5'-encoded isotypes or all the 3'-encoded isotypes in fragments secreting a defined class of Ab. This comparison clearly shows that there is a higher frequency of co-expression of all the studied 5'-encoded Ig classes

TABLE VII
*Association of the Expression of a Given Isotype of Ab in TNP-Ficoll-stimulated Fragments with the Co-expression of Ab of All Isotypes Encoded by 5' Igh-C Genes**

Designated isotype	Frequency of total fragments positive for designated isotype (positive/total tested)	χ^2 ‡	Frequency of co-expression of all 5'-encoded isotypes	Frequency of co-expression of all 3'-encoded isotypes
IgM	105/427	—	—	8/105
IgG ₃	74/427	219	68/74	8/74
IgG ₁	26/427	99	22/26	8/26
IgG _{2b}	33/427	137	16/33	10/33
IgG _{2a}	13/427	133	8/13	—

* The fragments included in this analysis are from recipients of B + T lymphocytes.

‡ The χ^2 statistic (26) given refers to the association of expression of the designated isotype with the co-expression of all other isotypes encoded by 5' *Igh- γ* genes. In each case this association is highly significant ($P \ll 0.001$).

than co-expression of all the studied 3'-encoded isotypes. The χ^2 statistic of association of the expression of the designated isotype with the co-expression of all other 5'-encoded isotypes is, in every case, highly significant. It can be noted that not every fragment which secretes a given class of Ab also secretes all the 5'-encoded isotypes; there are exceptions to the trend. Analysis of these variants showed no consistent lack of production of a particular 5'-encoded isotype, with the exception of IgG₁ co-expression in IgG_{2b}-secreting clones. It is possible that, in these variant clones, switches to the nonsecreted isotype gene were very transient or else randomly bypassed.

That anti-TNP Ab of the IgG_{2a} subclass is almost entirely found in fragments that secrete IgM as well as most of the other classes of Ig strongly suggests that most IgG_{2a}-positive fragments arise from IgM-positive precursors and that clones that switch into IgG_{2a} production have, in the course of their expansion, also switched into production of the other 5'-encoded isotypes.

Early Requirement for T Cells for Optimum IgG_{2a} Enhancement. The data obtained from the splenic focus assay are compatible with the hypothesis that the preferential T cell enhancement of IgG_{2a} Ab synthesis to TNP-Ficoll is mediated by an amplification of the isotype-switching mechanism within B cell clones. This is based on the observations that T cells increase the frequency of B cell clones that secrete IgG_{2a} and that IgG_{2a}-positive clones appear to be actively switching clones, i.e., clones that have produced IgM as well as all or nearly all other IgG subclasses. Nevertheless, our observations do not exclude the possibility that the preferential enhancement of IgG_{2a} synthesis to TNP-Ficoll is mediated by IgG_{2a} isotype-specific T cells that act on B cells that have, by a T-independent mechanism, previously switched into IgG_{2a} production. It is possible that switches to IgG_{2a} occur with equivalent frequency in the presence or absence of T cells but that IgG_{2a} isotype-specific T cells are required for the initiation of IgG_{2a} secretion from membrane IgG_{2a}-positive cells. We have, therefore, taken two additional approaches to test for possible involvement of IgG_{2a} isotype-specific T cells.

Since IgG-positive daughter cells of antigen-specific precursors do not appear until

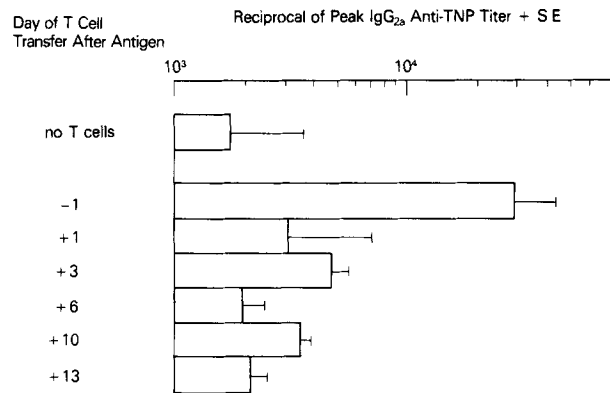


FIG. 2. Early requirement for T cells for optimum enhancement of the IgG_{2a} response to TNP-Ficoll. 10^7 nonprimed C57BL/10 ScN *nu/+* purified splenic T cells were injected intravenously into C57BL/10 ScN *nu/nu* mice at various times before or after TNP-Ficoll immunization. Each group consisted of four mice.

several days after immunization (27), we predicted that IgG_{2a}-specific T cells would be unable to exert their helper effect until several days after the commencement of the response. Thus, isotype-specific T cells might be expected to function equally well when transferred 1 d before antigen (as in previous experiments) or up to several days after antigen. Fig. 2 shows the data from an experiment in which comparisons were made of the ability of T cells to enhance IgG_{2a} synthesis when transferred at various times before or after antigen. Significant enhancement of the IgG_{2a} response was obtained only when T cells were transferred 1 d before immunization (data from another experiment indicates that T cells are also effective when transferred at the same time as antigen; data not shown). Transfer of T cells from 1 to 13 d after antigen was administered failed to significantly enhance the peak IgG_{2a} response above that measured in nonreconstituted *nu/nu* mice.

Ability of T Cells from Neonatally Anti-IgM-suppressed Mice to Enhance TNP-Ficoll-induced IgG_{2a} Synthesis. A number of reports have suggested that immunoglobulin-specific T cells are absent in mice whose immunoglobulin production has been suppressed by treatment with anti-IgM since birth (28–30). To determine whether the T cell responsible for enhancing IgG_{2a} Ab synthesis to TNP-Ficoll might share this property, we compared the ability of T cells from normal or from neonatally anti-IgM-suppressed mice to enhance the IgG_{2a} production of *nu/nu* mice to this antigen. The data in Table VIII clearly show that purified T cells from anti-IgM-suppressed mice were as efficient at enhancing IgG_{2a} synthesis as normal T cells.

Discussion

In this report we have shown that the athymic *nu/nu* mouse response to the TI-2 antigen, TNP-Ficoll, is characterized by a distinct hierarchy of Ab production of IgM and the various IgG subclasses. This hierarchy was evident after (a) analysis of serum levels of the various isotypes of anti-TNP Ab, (b) quantitation of the *in vitro* secretion of Ab from *in vivo* immunized spleen cells, and (c) measurement of the frequency of TNP-Ficoll-responsive B cell clones that secrete Ab of the various isotypes in a splenic focus assay. An analysis of the plaque-forming cell response of normal euthymic mice

TABLE VIII
Ability of T Cells from Anti-IgM-suppressed Mice to Enhance IgG_{2a} Response to TNP-Ficoll

Mice tested*	Reciprocal of peak anti-TNP serum titer	
	IgG ₁	IgG _{2a}
<i>nu/nu</i>	5,939 \bar{X} 1.48	26 \bar{X} 1.98
<i>nu/nu</i> + T cells	3,356 \bar{X} 1.26	2,211 \bar{X} 1.46
<i>nu/nu</i> + Anti-IgM-suppressed T cells	1,437 \bar{X} 1.39	2,362 \bar{X} 1.11

* C57BL/10 ScN *nu/nu* mice were given no T cells or injected with 5×10^6 splenic T cells from untreated *nu/+* mice or 5×10^6 splenic T cells from neonatally anti-IgM suppressed mice. T cells were transferred 1 d before TNP-Ficoll immunization. Each group consisted of four mice. Data are expressed as the geometric \bar{X} of reciprocal serum titers \bar{X} SE.

to TNP-Ficoll by Slack et al. (31) is consistent with this isotypic order. The isotypic hierarchy we have observed is of particular interest because it directly correlates with the 5' to 3' *Igh-C* gene order (5' *Igh- μ* , *Igh- γ 3*, *Igh- γ 1*, *Igh- γ 2b*, *Igh- γ 2a* 3') (9, 10) and suggests that the position of a given *Igh- γ* gene on the chromosome may influence the rate at which switching to that gene may occur in a B cell clone that responds to TNP-Ficoll. In addition, we have further confirmed our previous observation (7) that T cells can preferentially enhance, in relative terms, IgG_{2a} antibody synthesis to TNP-Ficoll and can thus sometimes alter the intrinsic isotypic production hierarchy characteristic of this type 2 antigen.

Two major explanations exist for the preferential T cell enhancement of IgG_{2a} Ab synthesis to TNP-Ficoll. The enhancement of IgG_{2a} Ab synthesis may be attributed to IgG_{2a} isotype-specific T cells that selectively expand the proliferation or the secretion rates of TNP-Ficoll-specific clonal progeny which have already switched to IgG_{2a}. Alternatively, T cells of yet undefined specificity, may simply enhance the switching process within stimulated B cell clones. Thus, they may preferentially encourage the switch of clonal progeny into those genes, e.g., *Igh- γ 2a*, which are located the furthest downstream from the switching region 5' to the *Igh- μ* gene (11, 12) and which may have a low probability of being switched to in the absence of T cell signals.

The data presented are more in keeping with the hypothesis that T cells preferentially increase IgG_{2a} anti-TNP-Ficoll Ab production through an enhancement of the switching process. The evidence supporting this hypothesis is best understood by first analyzing the repertoire of isotypes produced in individual B cell clones responding to TNP-Ficoll. Results from the splenic focus assay demonstrate that the majority of clones secreting IgG isotypes also secrete IgM. These findings strongly suggest that most of the clones that secrete multiple isotypes probably originated from a single IgM-positive precursor. This is consistent with the observation that B cells that produce IgG after lipopolysaccharide (LPS) stimulation originate from IgM precursors (32, 33). Since in the B cell response to TNP-Ficoll in the absence of T cells the hierarchy in quantitative production of the various IgG subclasses and the frequency of clones secreting each of these isotypes follows the 5' to 3' *Igh-C* gene order, it is tempting to conclude that, within an expanding B cell clone derived from a single

IgM-positive precursor, switches to *Igh- γ* genes located the greatest distance downstream from the *Igh- μ* gene may have lower probabilities of occurring than switches to more proximal *Igh- γ* genes. Furthermore, the observation that clones that produce IgG_{2a} have produced all or nearly all of the other IgG isotypes suggests that switches to the most distal *Igh- γ* gene, *Igh- γ 2a*, are preceded or accompanied by switches to all of the 5'-encoded *Igh- γ* genes. In fact, the trend in our data has shown that clones that switch into the production of any given IgG isotype tend to secrete 5'-encoded isotypes with a much higher frequency than 3'-encoded isotypes. These results do not necessarily imply the occurrence of sequential switching events within a lineage of daughter cells (34). They could as easily be explained by single $\mu \rightarrow \gamma 3$, $\mu \rightarrow \gamma 1$, $\mu \rightarrow \gamma 2b$, and $\mu \rightarrow \gamma 2a$ switches occurring within separate daughter cells of a clone. The statistical incidence of each of these switches might be influenced by the length of DNA over which such gene rearrangements must occur.

Our observations that T cells enhance predominantly the in vivo production of those isotypes which, in the absence of T cells, are produced in the lowest amounts in itself suggested that T cells may increase the frequency of *Igh-C* gene switches which, in the absence of T cells, have low incidence of occurrence. This conclusion is strongly supported by the observation that T cells can significantly increase the frequency of B cell clones which produce IgG_{2b} and IgG_{2a} to TNP-Ficoll in the splenic focus assay. Since IgG₂-positive clones appear to be clones which are actively switching, it is very likely that T cells that increase the frequency of IgG₂-positive clones do so by enhancing the ability of B cell clones that are already switching to the *Igh- γ 3* and *Igh- γ 1* genes to also switch downstream to the *Igh- γ 2b* and the *Igh- γ 2a* genes. This interpretation is compatible with the recent observations by Martinez-Alonso et al. (35), which suggest that T cells can cause a 5' to 3' directional switch in isotype expression within a mitogen-stimulated B cell population.

Contrary to the evidence consistent with T cell enhancement of the switching process, we have found no evidence in support of IgG_{2a}-isotype-specific T cells. The evidence against them is indirect but is as follows: (a) Mice that have been suppressed in Ab production by treatment with anti-IgM since birth have T cells that can enhance IgG_{2a} Ab synthesis. In contrast, a number of other reports have shown that putative Ig-specific T cells are absent in anti-IgM suppressed mice (28-30). (b) The T cells responsible for enhanced IgG_{2a} synthesis are required early in the response to TNP-Ficoll, i.e., on the day of antigen stimulation or earlier. In sheep erythrocyte antigen stimulation, daughter cells bearing IgG do not appear until day 3 of the response (27). If TNP-Ficoll-responsive B cell precursors differentiate in a similar manner, then one might expect isotype-specific T cells that function by recognizing B cell membrane IgG_{2a} to be effective even when transferred as late as 3 d after antigen stimulation. This was not the case; T cells transferred even 1 d after TNP-Ficoll were relatively ineffective at enhancing the IgG_{2a} Ab response when compared with T cells transferred 1 d before the antigen. (c) The T cell responsible for preferential enhancement of the IgG_{2a} response to TNP-Ficoll is found in allotype-congenic B.C8 mice. (P. K. A. Mongini, D. Longo, and W. E. Paul. T cell regulation of immunoglobulin class expression in the B cell response to TNP-Ficoll: characterization of the T cell responsible for the selective enhancement of the Ig_{2a} response. Manuscript in preparation). Thus, if the T cell is IgG_{2a} specific, it is not specific for allotypic determinants, as is the IgG_{2a}-specific helper T cell observed by Herzenberg

et al. (5). (d) Although T cells predominantly affect the production of IgG_{2a}, they can also somewhat affect the production of other subclasses. This lack of an absolute specificity does not favor the existence of a unique IgG_{2a}-isotype-specific helper T cell.

In summary, we feel that the bulk of our observations supports the hypothesis that syngeneic unprimed T cells can modulate the isotypic response of TNP-Ficoll-activated B cells through an influence on the switching process. This has not been unequivocally proven, however. For instance, we cannot exclude the possibility that switches to *Igh-γ2a* occur with equivalent frequency in the presence or absence of T cells but that IgG_{2a} secretion may not occur without accessory T cell signals. We think this possibility unlikely because, first, it is difficult to reconcile a strong dependence of IgG_{2a} secretion on T cells with the relationship that IgG_{2a} production has with *Igh-C* gene order, and second, because some IgG_{2a} Ab secretion does occur in the absence of thymus-processed T cells. In addition, we cannot exclude the possibility that in the presence of T cells, a new set of TNP-specific B cell clones that requires T cell signals for activation express themselves. These T-dependent B cell clones may more easily switch into IgG_{2a} production than T-independent B cell clones.

We have here and elsewhere stressed the influence that *Igh-C* gene order has on the quantities of the various isotypes of Ab produced in the B cell response to TNP-Ficoll. Although another TI-2 antigen that stimulates the Lyb-3⁺5⁺ B cell subpopulation, TNP-levan, has been shown to stimulate a similar isotypic response (7), the TI-2 antigen dextran, antigens such as TNP-*Brucella abortus* and TNP-LPS (which we would now designate as type 1 antigens), and classical T-dependent antigens can stimulate different isotypic responses (31, 36, 37; and Dr. Kathryn Stein, personal communication). Clearly there may be factors in addition to 5' to 3' *Igh-C* gene order that affect the representation of the various isotypes of Ab in B cell responses to antigens. The polyclonal activation signals associated with most type 1 antigens, signals from T cells, the subpopulation of B cells being stimulated, and the existence of memory cells through previous exposure to environmental antigens may all influence the isotypic repertoire of B cell clones responding to antigen. We propose that the isotype switching events that take place during the B cell response to TNP-Ficoll, in the absence of thymus-matured T cells, represent an intrinsic switching mechanism for IgG subclass genes in the Lyb-3⁺5⁺ B cell subpopulation. In this process, the order of *Igh-C* genes on the chromosome may play a considerable role in influencing the likelihood that a particular IgG gene product will be produced. Additional signals provided by T cells, macrophages, or polyclonal activators may modify this intrinsic isotypic response hierarchy.

In this report, we have examined the response of isotypes encoded by genes to the 3' end of the *Igh-C* gene complex, i.e., IgM, IgG₃, IgG₁, IgG_{2b}, and IgG_{2a}. Two other *Igh-C* genes, *Igh-ε* and *Igh-α*, have been localized to sites downstream from the *Igh-μ* and *Igh-γ* genes (9, 10). If the chromosomal position of these genes has an influence on the probability of switches to IgE and IgA production, we would predict that the amount of Ab of these isotypes produced in TNP-Ficoll-stimulated *nu/nu* mice should be even lower than the amount of IgG_{2a} produced. Our unpublished results (P. K. A. Mongini, W. E. Paul, and E. S. Metcalf. Analysis of in vivo and in vitro IgE and IgA responses to the TI-2 antigen, TNP-Ficoll. Manuscript in preparation) indicate that although this appears to be the case when in vivo responses are measured, in the in vitro splenic focus assay a much higher proportion of fragments secrete IgE and IgA

antibody than would be predicted by the position of their respective *Igh-C* genes on the chromosome. Thus, the switch to *Igh-ε* and *Igh-α* genes may be under a separate type of control. It is possible that within the Lyb-3⁺5⁺ B cell stimulated by TNP-Ficoll, three unique IgG-, IgE-, and IgA-specific switching enzymes are generated that induce switching to each of the *Igh-γ* genes, to *Igh-ε*, and to *Igh-α*, respectively. Indeed, IgA-specific switching sites have been reported and the existence of class-specific switching enzymes has been proposed (11).

Although we have clearly shown that T cells can preferentially increase the amount of certain IgG subclasses of Ab produced to TNP-Ficoll, and although we have shown that T cells may very likely do this by influencing the *Igh-C* gene switching process within TNP-Ficoll-responsive B cell clones, we have not addressed the specificity of the T cell responsible nor the mechanism by which T cells influence the switch to *Igh-γ2b* and *Igh-γ2a* genes. Experiments designed to study the specificity question will be presented elsewhere. With regard to the mechanism of T cell influence on IgG_{2a} production, it is possible that T cells enhance isotype switching events by enhancing the proliferation of TNP-Ficoll-responsive B cell clones. In fact, previous data have suggested that the commencement of IgG synthesis after antigen or mitogen stimulation is accompanied by and may require B cell proliferation (38, 39). As an alternative explanation, T cell signals may increase the production of an IgG-specific switching enzyme and thus mediate an increase in the frequency of relatively low-probability rearrangements, e.g., the switch to the *Igh-γ2a* gene. A thorough understanding of the mechanism of T cell enhancement of the switching process will undoubtedly be facilitated by an in vitro system in which switching events can be induced within a homogeneous population of B cells.

Summary

In the absence of T cells, B cells were found to respond to the type 2 T-independent (TI-2) antigen, trinitrophenyl (TNP)-Ficoll, with a characteristic hierarchy of IgM and IgG subclass Ab production which directly correlated with 5' to 3' *Igh-C* gene order, i.e., IgM > IgG₃ > IgG₁ > IgG_{2b} > IgG_{2a}. This was evident when immune serum Ab titers were analyzed, when in vitro secretion of antibody from immune cells was measured and when TNP-Ficoll-stimulated clones in a splenic focus assay were analyzed for isotype production. T cells were found to cause a preferential relative increase in the amount of IgG_{2a} antibody produced to TNP-Ficoll. The T cell responsible was present in anti-IgM neonatally suppressed mice and was needed early in the response, i.e., on the day of immunization or earlier. T cells were found to increase the frequency of TNP-Ficoll-responsive B cell clones that produced IgG_{2a} in the splenic focus assay. The great majority of these IgG_{2a}-positive clones also produced IgM and all or nearly all of the IgG isotypes whose genes are encoded 5' to the *Igh-γ2a* gene. The data are discussed in terms to T cell enhancement of IgG_{2a} Ab synthesis being mediated through T cell enhancement of the *Igh-C* gene switching mechanism within TNP-Ficoll-responsive B cell clones. Thus, isotypes encoded by genes on the 3' end of the *Igh-γ* gene complex, which in the absence of T cells have a low probability of being switched to, are the most influenced by T cell help.

We thank Ms. Moira LaVeck for her expert technical assistance, Dr. David Alling for his help in generating a program to evaluate anti-TNP Ab titer in our RIA and for his help in the

statistical evaluation of our data, Dr. Alfred Singer for his critical review of this manuscript, and Ms. Shirley Starnes for her help in the preparation of the manuscript.

Received for publication 9 November 1981.

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