

INHIBITION OF T-ANTIGEN-BINDING CELLS BY IDIOTYPIC ANTISERA*

BY C. A. PRANGE, J. FIEDLER, D. E. NITECKI, AND C. J. BELLONE

(From the Department of Microbiology, St. Louis University School of Medicine, St. Louis, Missouri 63104; and the Department of Microbiology, University of California at San Francisco, San Francisco, California 94143)

It is well documented that known classes of immunoglobulin (Ig) on the surface of B cells serve as the receptors for antigenic determinants (1, 2). Published data concerning the T-cell recognition unit, however, leave its nature open to question. Several lines of evidence, i.e. antigen-binding data (3, 4) and studies of humoral T-cell factors (5), indicate that IgM serves as the receptor. In contrast, reports have shown that anti-Ig reagents neither block T antigen-binding cells (ABC)¹ (6) nor inhibit antigen-induced T-cell proliferation (7, 8). In the latter experiments, antisera directed against the product(s) of 2/13 histocompatibility locus in guinea pigs or product(s) of the *H-2* histocompatibility locus in mice effectively abrogate this response. More recent data in mice and guinea pigs indicate the inhibitory activity in the anti-histocompatibility sera are directed to Ia determinants found on the surface of T lymphocytes (9, 10). The above data do not rule out the possibility of either an entire Ig or some part thereof, i.e., the variable (V) region functioning as the antigen recognition unit.

Over the past few years, data from several laboratories indicate that T cells employ V regions of Ig as the antigen recognition site. The approach utilizes idiotypic antisera generated to isolated antibody (Ab) or to T-cell-bound receptors (11, 12). These idiotypic reagents have been able to block several T-cell functions (12, 13), stimulate both helper (11) and suppressor activity (14, 15), and enumerate the number of alloreactive T lymphocytes which express a given idiotypic (16).

We have recently reported a clonal expansion of Ig⁺ and Ig⁻ lymphocytes in L-tyrosine-*p*-azophenyltrimethylammonium [tyr(TMA)]-immune guinea pigs in the apparent absence of antibody production (17). If the monovalent tyr(TMA) antigen is made bivalent [H-L-tyr(TMA)-NH-(CH₂)₃]₂, separated by a nonimmunogenic spacer molecule, 1,6-diamino hexane, then antibody and cellular immunity specific for the tyr(TMA) moiety are consistently induced (18). This well-defined antigen system offers a unique opportunity to analyze antigen-specific

* Supported by NIH grants AI 13115 and AI 05664 from the U. S. Public Health Service and by the National Foundation-March of Dimes.

¹ Abbreviations used in this paper: Ab, antibody; ABA_{9,5}-BSA; azobenzene-*o*-arsenate-bovine serum albumin; ABC, antigen-binding cells; anti-Id, idiotypic antiserum; CFA, complete Freund's adjuvant; DMF, dimethylformamide; DPBS, Dulbecco's phosphate-buffered saline; FCS, fetal calf serum; LNC, lymph node cells; NGPIg, normal guinea pig Ig; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; t-Boc, *N*-*t*-butyloxycarbonyl; t-Boc-L-TMD, bis-*N*-*t*-Boc-L-tyrosylhexamethylenediamide; TMA₁₀-BSA, azophenyltrimethylammonium-bovine serum albumin; TMA-S₁₅-BSA, 3-(*p*-trimethylaminophenylazo)-*N*-acetyl-L-tyrosylglycylglycine-bovine serum albumin; tyr(ABA), L-tyrosine-*p*-azobenzene-*o*-arsenate; tyr(TMA), L-tyrosine-*p*-azophenyltrimethylammonium; V region, variable region.

T- and B-cell receptors for shared V regions. We now present evidence which suggests that T and B cells utilize the same V region as judged by shared idiotypy. In addition, these data indicate that V regions on T cells are not linked to Ig constant regions.

Materials and Methods

Animals. Both outbred albino and inbred strain 13 guinea pigs of both sexes weighing 300–400 g were used throughout these experiments. Outbred guinea pigs were purchased from Isaacs Lab Stock, Inc., Litchfield, Ill. while the strain 13 guinea pigs were obtained from the University of Missouri, Columbia, Mo. and from Biological Systems, Toms River, N. J. New Zealand white rabbits were purchased from the Eldridge Laboratories, Barnhart, Mo.

Antigens. The monovalent antigens tyr(TMA) and L-tyrosine-*p*-azobenzene-*o*-arsenate [tyr(ABA)] were prepared as previously described (17, 19). The bivalent antigen [(H-L-tyr(TMA)-NH-(CH₂)₃)₂] was prepared by reacting two equivalents (50 mmol) of *N*-*t*-butyloxycarbonyl *t*-Boc-L-tyrosine-*N'*-hydroxysuccinimide ester (Bachem, Inc., Marina Del Rey, Calif.) with one equivalent of 1,6-diamino hexane (25 mmol) in a total vol of 600 ml of dimethylformamide (DMF) for 72 h at room temperature. The desired product bis-*N*-*t*-Boc-L-tyrosylhexamethylenediamide (*t*-Boc-L-TMD) was extracted several times into a total of approximately 1.3 liters of ethylacetate, vacuum dried, and further purified by repeated recrystallization in methanol. 2.5 g of this material (~1 equivalent) was dissolved in 50 ml of DMF and 20 ml of H₂O and then reacted with two equivalents of the diazonium salt of *p*-trimethylaminoaniline at 4°C overnight. Reaction of *t*-Boc-L-TMD with two equivalents of diazotized *p*-trimethylaminoaniline yields a mixture of derivatives. The bis-substituted phenols exhibit deep purple color; mono-substituted phenols are orange. The desired product [t-Boc-L-tyr(TMA)-NH-(CH₂)₃]₂ was isolated by repeated chromatography on silica gel columns in a chloroform-methanol-ammonium hydroxide solvent (15:20:10). The *N*-protecting *t*-butyloxycarbonyl groups were removed by formic acid, yielding the expected product [H-L-tyr(TMA)-NH(CH₂)₃]₂. The bifunctionality was established by UV spectroscopy of the azochromophore and by high voltage electrophoresis. Purity of all intermediates and the final product were investigated extensively by thin-layer chromatography on silica gel and cellulose in several solvent systems.

The hapten protein conjugate 3-(*p*-trimethylaminophenylazo)-*N*-acetyl-L-tyrosylglycylglycine-bovine serum albumin (TMA-S₁₅-BSA) was prepared according to the methods of Inman et al. (20). Compound (M), tyr(TMA) glycyglycine Boc hydrazide, was a generous gift of Dr. John K. Inman, NIH, Bethesda, Md. The conjugates azobenzene-*o*-arsenate-bovine serum albumin (ABA_{9,5}-BSA), and azophenyltrimethylammonium-bovine serum albumin (TMA₁₀-BSA) were prepared according to the methods of Tabachnick and Sobatka (21). Twice crystallized BSA was purchased commercially (Sigma Chemical Co., St. Louis, Mo.).

Immunization. Tyr(TMA), tyr(ABA), and the bivalent antigen [H-L-tyr(TMA)-NH-(CH₂)₃]₂ were dissolved in 0.1 N NaOH and quickly diluted with phosphate-buffered saline (PBS), the pH was adjusted to neutrality with HCl, and the aqueous solution was emulsified with equal volumes of complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, Mich.). Both tyr(TMA)- and tyr(ABA)-immune animals were inoculated with 400 μg of the antigen distributed between the four foot pads. 400 μg of the bivalent antigen were distributed between the four foot pads, and the guinea pigs boosted subcutaneously thereafter seven to eight times with 400 μg of antigen over a 12-mo period. Antiserum was regularly collected throughout this 12-mo period. TMA-S-BSA was dissolved in PBS and mixed with an equal volume of CFA and immunized as described for the bivalent antigen except that 100 μg of antigen was used in the priming and booster inoculations.

Immunoabsorbents. Sepharose normal guinea pig immunoglobulin (NGPIg) was prepared by reacting 20–30 mg of an 18% Na₂SO₄ precipitate fractionated from normal strain 13 guinea pig serum with 10 ml of packed cyanogen bromide-activated (22) Sepharose 4B (Sigma Chemical Co.). Rabbit anti-polyvalent guinea pig Ig and normal rabbit serum were prepared by reacting 5 ml of a 1:10 dilution of serum (in sodium citrate buffer, pH 6.5) with 10 ml of packed cyanogen bromide-activated Sepharose 4B. Sepharose-tyr(TMA) immunoabsorbent was prepared according to the procedures of Cheseboro and Metzger (23). The idiotypic antiserum (anti-Id)-Sepharose was prepared by reacting 5 ml of a 1:10 dilution of antiserum with *N*-hydroxysuccinimide-activated Sepharose 4B according to the methods of Gottlieb et al. (24).

Preparation of Anti-Id. Anti-tyr(TMA) Ab was isolated by affinity chromatography using a tyr(TMA)-Sephacrose column. After elution of the Ab with 10^{-3} M tyr(TMA), the protein was exhaustively dialyzed against borate-buffered saline, pH 8.0, followed by dialysis against a 0.01 M KH_2PO_4 buffer, pH 4.7, and finally passed over a Dowex 50W \times 8 column (Dow Chemical Co., Midland, Mich.) equilibrated in the same solution to eliminate dissociable tyr(TMA) from the isolated antibody (25). Eluted antibody was analyzed on 10% sodium dodecyl sulfate (SDS) polyacrylamide gels under reducing conditions according to the procedures of Laemmli (26). 50 μg of this material gave four bands of which three comigrate with either heavy or light chains of guinea pig IgG and IgM. The fourth band comigrated with an isolated guinea pig albumin preparation (Sigma Chemical Co.).

Absorption of this material with either the anti-Ig or the anti-Id immunoabsorbents removes three of the four bands, leaving the albumin-migrating band intact (figures not shown). These absorption experiments indicate no detectable tyr(TMA)-binding factors other than Ig comigrate in polyacrylamide gel electrophoresis with either heavy or light chains. Unabsorbed isolated antibody was mixed with Sepharose coupled to normal rabbit serum and run in parallel with specific absorption procedures to control for nonspecific loss.

500 μg of the antibody in CFA was inoculated into each of several rabbits. Antiserum was collected 30–40 days later, and only sera which gave a strong precipitin reaction in agarose gels against the isolated antibody were used. The rabbit antisera were then absorbed with NGPIg-Sepharose and then tested for idiotypic specificity in a radio immunoassay as described (27).

The two anti-idiotypic sera used in this report were each prepared from pooled guinea pig antisera coming from a single strain 13 animal either inoculated with [H-L-tyr(TMA)-NH-(CH_2)₃]₂, [rabbit anti-Id(TST)], or TMA-S-BSA, [rabbit anti-Id(TSB)] (see immunization protocol).

Iodination. ^{125}I -TMA₁₀-BSA, ^{125}I -ABA_{9.5}-BSA, and isolated ^{125}I -anti-tyr(TMA) Ab were prepared by the chloramine-T method (28). Several preparations of ^{125}I -TMA₁₀-BSA and ^{125}I -anti-tyr(TMA) were used with specific activities of the preparations ranging from 12 to 20 $\mu\text{Ci}/\mu\text{g}$. The one preparation of ^{125}I -ABA-BSA had a specific activity of 15 $\mu\text{Ci}/\mu\text{g}$.

Assay for ABC. Detection of antigen bound to cells was performed by methods as previously described (17, 29). Briefly, ^{125}I -TMA-BSA was added to 20–40 \times 10⁶ cells in 0.5 ml of minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) (Grand Island Biological Co., Grand Island, N. Y.), and containing 1 mg/ml of sodium azide. Inhibition studies were carried out by adding the appropriate inhibitors (see Results section) 30 min before and during incubation of cells at 4°C with ^{125}I -TMA-BSA. All binding experiments were carried out at 4°C except where stated in the Results section. After 30 min incubation at 4°C, the cells were washed once in Dulbecco's phosphate-buffered saline (DPBS) (GIBCO), pH 7.4, and once over a 3 ml cushion of FCS. Cells were resuspended in DPBS and reacted with fluorescein-conjugated rabbit anti-guinea Ig (Miles Laboratories, Inc., Miles Research Products, Elkhart, Ind.) and washed twice over FCS cushions. The cells were then smeared on gelatin-coated slides, air dried, fixed in 95% EtOH, dipped in Kodak NTB-2 emulsion (Eastman Kodak Co., Rochester, N. Y.), and exposed for 5 days at 4°C except for one group of experiments where the autoradiographs were exposed for 14 days (see Results section). Development was in Kodak D-19 developer. ABC were identified as cells with five or more silver grains on their surface using phase contrast on a Leitz Ortholux II microscope (E. Leitz, Inc., Rockleigh, N. J.). After an ABC was found, an ultraviolet light source was applied, the cell evaluated for fluorescence, and scored as either surface Ig⁺ or Ig⁻.

Class-Specific Antisera. Preparation of class-specific antisera used in blocking experiments was described in a previous report (17). All antisera used, including the anti-Id, were concentrated three times by 18% Na_2SO_4 fractionation, and dilutions (see Results section for final dilution) from these Ig-enriched concentrates were then used in the blocking experiments.

Trypsinization and Resynthesis of T-Cell Receptors. Briefly, 20–40 \times 10⁶ cells were suspended in 1 ml of MEM containing 150 $\mu\text{g}/\text{ml}$ trypsin (Worthington Biochemical Corp., San Francisco, Calif.) three times crystallized and 100 $\mu\text{g}/\text{ml}$ DNase I (Worthington Biochemical Corp.). After incubation for 45 min at 37°C, the cells were washed three times in MEM-5% FCS, and were then resuspended in resynthesis media consisting of MEM supplemented with 5% FCS, 2 mM glutamine, 100 U/ml of penicillin G, and 50 $\mu\text{g}/\text{ml}$ streptomycin. After incubation for 16 h, the cells were harvested, washed, and the ABC assay was performed.

Results

Characterization of Guinea Pig Ig⁺ and Ig⁻ ABC. In a previous report we demonstrated that after immunization with tyr(TMA) in CFA specific ABC expanded seven- to ninefold over the CFA control population (17). This clonal proliferation occurred in both the Ig⁺ (60% of the total) and Ig⁻ (40%) lymphocyte populations. In order to study shared idiotypity on T- and B-ABC it was necessary to further characterize the Ig⁺ and Ig⁻ populations to be reasonably certain we were dealing with B and, in particular, T cells in the guinea pig species. Accordingly, we tested a number of parameters reported to be characteristic of T- and B-ABC in the better defined mouse species (6, 30). As seen in Table I the frequency of TMA-BSA ABC in immune animals as compared to adjuvant-primed controls increases eight- and sevenfold in the Ig⁺ and Ig⁻ populations, respectively, at 4°C. Approximately three times as many Ig⁻ ABC were detected when the incubation with antigen was done at 37°C rather than 4°C. In contrast, antigen-binding Ig⁺ cells were found not to be significantly affected by temperature. In addition, this increase of Ig⁻ ABC at 37°C is inhibited by including sodium azide in the incubation medium. The results in Table I also show the importance of the length of exposure time of autoradiographs for 14 instead of 5 days, the number of Ig⁻ ABC was increased by slightly more than twofold while the frequency of Ig⁺ ABC was virtually unaffected.

To further study the two populations, a comparison of the avidity of receptors on ABC was determined by including tyr(TMA) at various concentrations in the medium with immune lymph node cells (LNC) and ¹²⁵I-TMA-BSA. As shown in Fig. 1, the value of tyr(TMA) inhibition for antigen-binding Ig⁺ and Ig⁻ LNC changes between 1-6 wk after immunization. At 1 wk both Ig⁺ and Ig⁻ ABC are of such low avidity that the vast majority of these cells cannot be inhibited by concentrations of tyr(TMA) as high as 10⁻³ M. The avidity of the cellular receptors changes until at 6 wk the I₃₀ of the Ig⁺ ABC is approximately three orders of magnitude lower than the concentration of tyr(TMA) required at 1 wk, while the I₃₀ of Ig⁻ ABC is only 1.5 orders of magnitude lower than 1 wk. While the Ig⁺ population undergoes a large change in avidity, the Ig⁻ lymphocytes undergo a lesser, but nevertheless, significant difference in overall avidity with time after immunization. The steep slope of the inhibition curves of Ig⁻ ABC suggest that this population is much more restricted than the Ig⁺ ABC population. This and previous data (17), in addition to further data presented in this paper, strongly suggest we are dealing with bona fide B (Ig⁺) and T (Ig⁻) ABC in this species.

Inhibition of ¹²⁵I-TMA-BSA Binding to T and B Lymphocytes by Class-Specific and Anti-Id. The nature of receptors on ¹²⁵I-TMA-BSA binding lymphocytes from tyr(TMA)-immune animals was investigated by reacting lymphocytes with idiotypic and class-specific antisera before and during exposure to ¹²⁵I-TMA-BSA. As shown in Table II, polyvalent anti-Ig and class-specific anti-IgM inhibit 100% and 90%, respectively, B-ABC in tyr(TMA)-immune guinea pigs while none of these anti-Ig reagents inhibit the T-ABC. In contrast, the two anti-Id preparations inhibit this latter population from 78 to 83% and the B-ABC from 50 to 55%. It is worth noting that the anti-Id preparations inhibited both strain 13 and outbred T- and B-ABC equally as well. These results with regard

TABLE I
Influence of Temperature and Sodium Azide on ^{125}I -TMA-BSA Binding by Ig^+ and Ig^- Cells

| Source of LNC | Antigen-binding temperature | Total ABC/ 10^4 | ABC/ 10^4 lymphocytes | |
|---------------|-----------------------------|-------------------|-------------------------|---------------|
| | | | Ig^+ | Ig^- |
| CFA | 4°C | 9 ± 0.25 | 5 ± 0.23 | 4 ± 0.04 |
| Tyr(TMA) | 4°C | 67 ± 4.3 | 39 ± 2.8 | 27 ± 2.1 |
| Tyr(TMA) | 37°C | 125 ± 8.0 | 43 ± 7.5 | 82 ± 10 |
| Tyr(TMA) | 37°C (NaN_3) | 72 ± 4.1 | 40 ± 5.5 | 32 ± 3.5 |

Effect of Exposure Time of Autoradiographs on the Frequency of Ig^+ and Ig^- ABC

| Length of exposure time | Total ABC/ 10^4 | ABC/ 10^4 Lymphocytes | |
|-------------------------|-------------------|-------------------------|---------------|
| | | Ig^+ | Ig^- |
| days | | | |
| 5 | 70 ± 10.4 | 48 ± 8.3 | 22 ± 3* |
| 14 | 104 ± 16 | 57 ± 9.1 | 47 ± 6 |

ABC were prepared as described in Materials and Methods. In CFA controls 5×10^5 total lymphocytes per animal were counted and expressed as ABC/ 10^4 lymphocytes counted. In immune animals 1×10^4 total lymphocytes per animal were counted. LNC were exposed to ^{125}I -TMA-BSA at 4°C with 1 mg/ml of sodium azide in the medium or at 37°C with or without azide. The number of ABC/ 10^4 lymphocytes represents the mean and standard error of 7-17 experiments.

* The difference between 47.5 ± 6 and 22 ± 3 is statistically significant ($P < 0.01$) as calculated by the Student's t test.

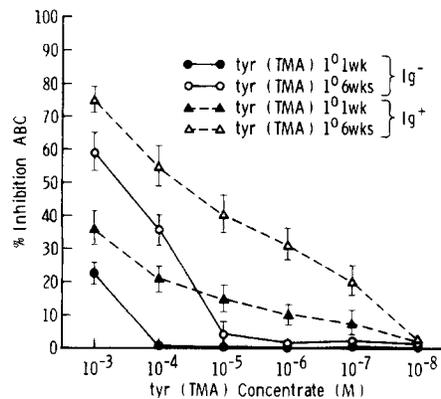


FIG. 1. ABC prepared as described (Materials and Methods) with concentrations of tyr(TMA) ranging from 10^{-3} to 10^{-8} M serving as inhibitor of ABC during exposure to TMA-BSA- ^{125}I . The avidity profile of: (●—●), Ig^- tyr(TMA) immune 1° 1 wk; (○—○), Ig^- tyr(TMA) immune 1° 6 wk; (▲—▲), Ig^+ tyr(TMA) immune 1° 1 wk; (△—△), Ig^+ tyr(TMA) immune 1° 6 wk is shown. % I = $(1 - [\text{tyr(TMA) treated/nontreated}] \times 100)$. Vertical bars represent the standard error. The difference between 1 and 6 wk in the Ig^- population at 10^{-3} M and 10^{-4} M are statistically significant ($P < 0.01$) as calculated by the Student's t test.

TABLE II
Inhibition of ¹²⁵I-TMA-BSA-Binding Ig⁺ and Ig⁻ LNC by Various Specific Antisera

| Antiserum | Total ABC/10 ⁴ | ABC/10 ⁴ lymphocytes | |
|-----------------------|---------------------------|---------------------------------|----------------|
| | | B-ABC | T-ABC |
| NRS | 74 ± 4.9 | 45 ± 3.9 | 29 ± 1.2 |
| Anti-Ig | 30 ± 1.6 | 0(100)* | 30 ± 1.5 (0) |
| Anti-IgM | 35 ± 1.9 | 5 ± 0.25 (90) | 30 ± 1.8 (0) |
| Anti-IgG ₁ | 71 ± 3.8 | 42 ± 3.5 (6) | 29 ± 1.4 (2.4) |
| Anti-IgG ₂ | 73 ± 4.2 | 44 ± 3.8 (2.1) | 29 ± 1.3 (1.0) |
| Anti-Id(TSB) | 25 ± 3.9 | 20 ± 3.5 (55) | 5 ± 0.9 (83) |
| Anti-Id(TST) | 29 ± 4.8 | 23 ± 4 (50) | 6 ± 1.2 (78) |

Guinea pigs (outbred albinos and strain 13) immunized 1-6 wk previously with 400 µg tyr(TMA) in CFA. ABC prepared as described in Materials and Methods except LNC reacted with antisera at a final dilution of 1:10 before and during exposure to ¹²⁵I TMA-BSA. Blocking data for B-ABC has been previously reported (17). ABC/10⁴ represent the mean and standard error of 8-18 experiments (number of ABC have been rounded off to the nearest integer). In each experiment 1 × 10⁴ total lymphocytes per animal were counted. NRS, normal rabbit serum.

* Number in parentheses = % inhibition (I) = [1 - (Ab treated/NRS) × 100].

to the B lymphocytes are consistent with the pattern of cross-reactive idiotypes (on the average of 60%) in serum from individual guinea pigs immunized with either the bivalent antigen [H-L-tyr(TMA)-NH-(CH₂)₃]₂ or TMA-S-BSA (data not shown). The restricted nature of the anti-TMA response in guinea pigs will be the subject of another manuscript which is in preparation. Since both anti-Id preparations, TST and TSB (see Materials and Methods for details) behave in an almost identical manner, inhibition experiments using these reagents will be averaged and shown as a single value for the remainder of the paper.

Specificity of the Anti-Id for the Tyr(TMA) Receptor. To demonstrate the specificity of the anti-Id for the tyr(TMA) receptor, guinea pigs were immunized with the small molecular weight antigen tyr(ABA). Several qualitative aspects of the immune response to the noncross-reacting antigen, tyr(ABA), and tyr(TMA) are virtually identical (31) and, thus, a good idiotypic specificity control. Although the number of ABA-specific ABC is half that of the tyr(TMA)-immune values, it is clear that the antisera do not inhibit tyr(ABA)-immune T and B cells from binding ¹²⁵I-ABA-BSA (see Table III).

Failure of NGPIg to Block the Inhibitory Properties of the Anti-Id. To further show the idiotypic specificity of our reagents, an Ig fraction containing at least IgG₂, IgG₁, and IgM was added at various concentrations with immune LNC and ¹²⁵I-TMA-BSA. As seen in Table IV, this Ig fraction at any concentration had no effect on the inhibition of T- and B-ABC by the anti-Id sera. In contrast, 100 µg of the fraction block 85% of the inhibitory capacity of the anti-Ig reagent for B-ABC. We have also shown in a radioimmunoassay that this Ig fraction does not inhibit the idiotypic antisera from binding homologous ¹²⁵I-anti-tyr(TMA) Ab (data not shown).

Effect of Trypsinization and Resynthesis of Surface Receptor on Nylon Wool-Passed ABC. To eliminate the possibility that cytophilic Ab is responsible for antigen binding in our T-ABC population, nylon wool-passed LNC were sub-

TABLE III
Specificity of Anti-Id for Receptors of Tyr(TMA)-Immune LNC

| Source of LNC | Antiserum | Total ABC/10 ⁴ | ABC/10 ⁴ lymphocytes | |
|---------------|-----------|---------------------------|---------------------------------|--------------|
| | | | B-ABC | T-ABC |
| Tyr(TMA) | — | 74 ± 4.9 | 45 ± 3.9 | 30 ± 1.2 |
| | Anti-Id | 27 ± 4.4 | 21 ± 3.7 (53)* | 6 ± 1.1 (80) |
| Tyr(ABA) | — | 43 ± 2.3 | 23 ± 1.2 | 20 ± 1.4 |
| | Anti-Id | 42 ± 3.0 | 22 ± 1.5 (5) | 21 ± 2.1 (0) |

Strain 13 guinea pigs immunized 1-6 wk previously with either 400 µg tyr(TMA) or tyr(ABA) in CFA. ABC prepared as described in Materials and Methods except LNC reacted with anti-Id at a final dilution of 1:10 before and during exposure to ¹²⁵I-TMA-BSA or ¹²⁵I-ABA-BSA. ABC/10⁴ represents the mean and standard of 4-10 experiments.

* Number in parentheses = % I = [1 - (Ab treated/normal rabbit serum) × 100].

TABLE IV
Failure of Free Ig to Block the Inhibitory Properties of the Anti-Id

| Immune | Antisera | Inhibitor | Total ABC/10 ⁴ | ABC/10 ⁴ lymphocytes | |
|----------|----------|--------------|---------------------------|---------------------------------|----------|
| | | | | B-ABC | T-ABC |
| Tyr(TMA) | — | — | 74 ± 12 | 47 ± 11 | 27 ± 2 |
| | Anti-Ig* | — | 26 ± 1.2 | 0 | 26 ± 1.2 |
| | Anti-Ig | 1 µg NGPIg‡ | 27 ± .9 | 0 | 27 ± .9 |
| | Anti-Ig | 10 µg NGPIg | 28 ± 1.5 | 1 | 27 ± 1.5 |
| | Anti-Ig | 100 µg NGPIg | 66 ± 3.6 | 39 ± 3.4 | 27 ± 1.4 |
| | Anti-Id | — | 21 ± 9 | 16 ± 8 | 5 ± 2 |
| | Anti-Id | 1 µg NGPIg | 21 ± 9 | 16 ± 8 | 5 ± 2 |
| | Anti-Id | 10 µg NGPIg | 22 ± 9.1 | 17 ± 8 | 5 ± 2 |
| | Anti-Id | 100 µg NGPIg | 22 ± 8.9 | 17 ± 8.4 | 5 ± 2 |

ABC/10⁴ represent the mean and standard error of four experiments.

* Both polyvalent anti-Ig and anti-Id were used at a final concentration of 1:10.

‡ NGPIg was obtained from an 18% Na₂SO₄ precipitate fractionation from normal strain 13 guinea pig serum. The various concentrations were added before and during exposure to the antisera and ¹²⁵I-TMA-BSA.

jected to trypsin treatment; cells were either immediately tested in the ABC assay or allowed to resynthesize their receptor and then assayed. As seen in Table V there is a 75% loss of tyr(TMA) ABC after trypsin treatment. If these treated cells are put into culture for 16 h and then assayed for ABC, 92% of the ABC are recovered. These cultured cells are inhibited by the anti-Id (77%) in a similar fashion to the untreated control indicating the re-expression of the same idiotypic population. Tyr(ABA)-immune LNC were treated in an identical fashion and as seen in Table V, trypsin treatment resulted in a 79% loss of ABC with a 95% recovery after the 16-h culture period. In addition, the failure of anti-Id to block these ABC further confirm the specificity of these reagents for the tyr(TMA) receptor.

Discussion

This present report indicates that V regions utilized by secreted and cell-

TABLE V
Effect of Trypsinization and Resynthesis of Surface Receptor of Column-Purified ABC

| Immune | Treatment | T-ABC/10 ⁴ lymphocytes | Loss of T- ABC* |
|----------|-------------------------------------|--------------------------------------|--------------------|
| | | | % |
| Tyr(TMA) | Normal rabbit serum | 39 ± 2.1 | |
| | Anti-Id | 9 ± 1.6 | 77 |
| | Trypsin-45 min | 10 ± .9 | 75 |
| | Trypsin followed by 16 h in culture | 36 ± 2 | 8 |
| | Trypsin 16 h culture + anti-Id | 9 ± 1.4 | 77 |
| Tyr(ABA) | Normal rabbit serum | 41 ± 8 | |
| | Anti-Id | 43 | 0 |
| | Trypsin 45 min | 9 ± 1 | 79 |
| | Trypsin followed by 16 h in culture | 39 ± 8.5 | 5 |
| | Trypsin 16 h culture + anti-Id | 38 ± 7.7 | 8 |

40 × 10⁶ column-purified LNC [according to methods of Julius et al. (32)] from tyr(TMA)- or tyr(ABA)-immune animals were incubated at 37°C for 45 min in 1 ml MEM containing 150 µg/ml trypsin, 100 µg/ml DNase I, and 5 mM MgCl₂. After trypsin treatments, cells were either immediately washed and subject to the ABC assay (± anti-Id) or allowed to resynthesize surface receptor by 16-h culturing before ABC determination. ABC/10⁴ lymphocytes represent the mean and standard error of two experiments in the tyr(ABA)-immune group and five experiments in the tyr(TMA)-immune animals.

* % loss of T-ABC = [1 - (treated population/normal rabbit serum) × 100].

bound Ig serve as part of the recognition unit on T cells. This evidence is based solely on cross-reactivity of the receptors as detected by anti-Id made to isolated serum anti-tyr(TMA) Ab. A key point in the interpretation of these results concerns the specificity of the anti-Id. In light of recent data, it is possible to coisolate by affinity chromatography Ig and other antigen-binding factor(s) from the serum (33). If our anti-Id contained Ab activity to different receptors which did not share idiotypy, then our interpretation is invalid. This problem has been circumvented in other systems by the use of the purified myelomas with known Ab activity (13). Since this is not possible in our system, we have subjected our serum tyr(TMA)-binding factors to biochemical and serological tests to characterize the nature of these components. Analysis on heavily loaded (see Materials and Methods) reducing SDS polyacrylamide gels (50 µg of protein/gel) revealed only four bands, three of which comigrate with heavy and light chains of guinea pig Ig and the fourth with an albumin marker. Furthermore, absorption experiments using either idiotypic or anti-Ig antisera coupled to Sepharose removed three bands, indicating that a possible T-cell factor(s) or components thereof having a molecular weight similar to Ig heavy or light chain was not detectably contaminating our Ig preparation.

Since a theta-like marker is not known for T cells in guinea pigs, evidence that we detect T-ABC must lie with indirect evidence. Data from our laboratory show that guinea pig Ig⁻ ABC resemble mouse T-ABC in several respects (6, 30, 32, 34). These include: (a) the absence of easily detectable surface Ig, (b) increased numbers of Ig⁻ ABC at 37°C (inhibited in presence of sodium azide), (c) longer exposure times of autoradiographs resulted in increased numbers of T-ABC, (d) passage through nylon wool, and (e) not inhibitable by a variety of

anti-Ig reagents. In addition, we demonstrate a marked avidity difference between the Ig⁺ and Ig⁻ ABC. At the very least, we can conclude that if our Ig⁻ ABC is not a T cell, we are looking at a most interesting antigen-specific cell type.

Our data fits well with a number of published reports dealing with the nature of the T-cell receptor. Other work has shown that antigen-specific proliferation in culture (35) and graft vs. host activity (12), both T-cell functions, can be inhibited by anti-Id. On the other hand, anti-Id have been shown to activate antigen-specific helper (11) and suppressor T-cell populations (14, 15). The above functional studies support the concept that T- and B-cell receptors share V regions, but it is not clear from this work whether the T-cell synthesizes this component. Our trypsinization and resynthesis experiments (see Table V) using column-purified LNC indicate that detected surface idiotypic determinants are synthesized by the T cell. We do not feel the re-expression of receptor is due to a B-cell cytophilic antibody product since, (a) we have virtually eliminated tyr(TMA)-specific B-ABC after column passage (<1/10⁴ lymphocytes, data not shown), and (b) essentially the same idiotypic population is re-expressed as judged by the blocking experiments. Regarding this latter point, we have demonstrated that tyr(TMA) B-ABC (and therefore receptors) are more heterogeneous than T-ABC as determined by hapten inhibition profiles (see Fig. 1). Thus, we feel it unlikely that any contaminating B cells after column passage would secrete the identical idio type found on T-ABC before trypsin treatment. This resynthesis data is in agreement with Binz and Wigzell (36) who have demonstrated by internal labeling experiments that an idio type-bearing molecule is synthesized by T cells.

The nature of the receptors on T cells other than its apparent antigenic similarity to V regions remains open to question. Our blocking experiments (Table II) suggest that the receptor-binding sites are not linked to classical Ig. We have not been able to inhibit the T-ABC with a polyvalent anti-Ig or class-specific antisera to IgM, IgG₁, or IgG₂. In contrast, these antisera effectively block tyr(TMA)-specific B-ABC. This negative inhibition data must be interpreted with caution since these anti-Ig reagents may not block, if in fact, class-specific determinants on Ig receptors are inaccessible to antisera due to receptor orientation in T-cell membranes. However, recent work in our laboratory (unpublished observations) does show that a 2 anti-13 strain antiserum (anti-Ia) inhibits T- but not B-ABC.² Our inhibition results with anti-Id and also with anti-Ia sera support several reports dealing with T-cell factors in mice (37, 38). These factors which are functionally active as helper and suppressor molecules are antigen specific, bear Ia determinants, but do not possess constant region determinants found on conventional Igs.

These results, as well as other mentioned idiotypic studies investigating the nature of the T-cell receptor are interesting in light of the altered-self hypothesis (39, 40). This hypothesis proposes that T cells recognize antigen-altered *H-2* specificities and not foreign determinants per se. This is clearly not the case

² Bellone, C. J., and C. A. Prange. 1977. Inhibition of T-antigen binding cells by idiotypic and Ia antisera. *In* ICN-UCLA Symposia on Molecular and Cellular Biology. E. E. Sercarz, L. A. Herzenberg, and C. F. Fox, editors. Academic Press, Inc., New York. In press.

with surface-bound or secreted Ig and thus difficult to reconcile with data indicating that T and B utilize the same V regions in antigen recognition. These apparent differences may be due, in part, to the nature of the different assays employed. Each test system, while providing insight, does not entirely explain the complex phenomenon of antigenic stimulation, i.e., antigen recognition and associative triggering signals. Perhaps part of the T-cell receptor recognizes foreign epitopes per se via V regions, and in addition, the receptor via another region of the molecule also recognizes altered *H-2* specificities. Both of these events would then be signals necessary for T-cell activation. The role of Ia determinants in this process remains to be determined.

An interesting sidelight from these studies which warrants some discussion comes from our results comparing the avidity of the T- and B-ABC receptors. In the course of the immune response to many antigens, there is an increase in the average affinity in serum antibody as well as the antigen-binding receptors on precursor B cells (41, 42). These affinity changes are believed to reflect an antigen driven selection among a population of precursor cells heterogeneous with respect to the affinity of their receptors. Whether a comparable maturation process occurs at the T-cell level is open to question. This is in part due to technical difficulties in demonstrating T-ABC using soluble antigens, and in particular, failure to detect "hapten"-specific T-ABC. As seen in Fig. 1, using hapten-inhibition techniques, there is a modest but significant increase in average avidity by Ig^- ABC for TMA-BSA. Earlier data, employing antigen-specific T-rosette assays (43) or lymphocyte transformation (44), suggest little or no affinity maturation at the T-cell level. Considering the assays and antigens employed, it is possible that a small but significant affinity maturation would go undetected. Most striking in the data (Fig. 1) is the difference in average avidity between the Ig^+ and Ig^- ABC populations. In addition, the slopes of the inhibition curves suggest a greater restriction in heterogeneity in the Ig^- population which is consistent with a small increase in average avidity. Since the tyr(TMA) Ig^- ABC have a markedly lower affinity for antigen, these data are also consistent with reports which indicate that T cells display a higher degree of specificity for antigen than serum Ab (30).

Summary

Shared idiotype between B- and T-cell receptors specific for the antigen L-tyrosine-*p*-azophenyltrimethylammonium [tyr(TMA)] was studied in an antigen-binding assay using idiotypic antisera. These idiotypic reagents were prepared by inoculation of rabbits with purified anti-tyr(TMA) antibody raised in strain 13 guinea pigs. The antisera blocked 78-83% of the antigen-binding T cells (T-ABC) and 50-55% of the antigen-binding B cells (B-ABC) from tyr(TMA)-immune strain 13 and outbred lymph node cells (LNC). An excess of normal guinea pig Ig in the ABC assay did not affect the ability of the idiotypic antisera to block T- and B-ABC. Nylon wool-passed tyr(TMA)-immune LNC were trypsin treated resulting in a 75% loss of T-ABC. The trypsin-treated population was then cultured for 16 h which resulted in a return of T-ABC to 92% of pretrypsin values. 77% of these regenerated T-ABC could be blocked with idiotypic antisera. Specificity of the idiotypic antisera was tested in L-tyrosine-*p*-

azobenzene arsonate-immune guinea pig LNC. Neither T- nor B-ABC were blocked in this heterologous system. Further blocking experiments were performed to characterize the nature of the T-ABC receptor. A variety of anti-Ig reagents, some of which block B-ABC, do not inhibit T-ABC suggesting that variable regions on T cells are not linked to Ig constant regions.

We wish to thank Dr. Joel Goodman for allowing Clifford J. Bellone to synthesize antigen in his laboratory. The expert technical assistance of Inge Stoltenberg as well as the secretarial assistance of Gail Pearse is gratefully appreciated. The authors wish to thank Dr. Eli Nadel and The McBride Love Foundation for support in the early steps of this project. Finally, we wish to thank Doctors Joseph M. Davie and Michael Green for their critical review of this manuscript.

Received for publication 2 May 1977.

References

1. Warner, N. L. 1974. Membrane immunoglobulins and antigen receptors on B and T lymphocytes. *Adv. Immunol.* 19:67.
2. Katz, D. H., and B. Benacerraf. 1972. Regulatory influence of activated T cells on B cell responses to antigen. *Adv. Immunol.* 15:1.
3. Roelants, G., L. Forni, and B. Pernis. 1973. Blocking and redistribution ("capping") of antigen receptors on T and B lymphocytes by anti-immunoglobulin antibody. *J. Exp. Med.* 137:1060.
4. Hogg, N. M., and M. F. Greaves. 1972. Antigen-binding thymus-derived lymphocytes. II. Nature of the immunoglobulin determinants. *Immunology.* 22:967.
5. Feldmann, M. 1972. Cell interactions in the immune response in vitro. V. Specific collaboration via complexes of antigen and thymus-derived cell immunoglobulin. *J. Exp. Med.* 136:737.
6. Kennedy, L. J., M. E. Dorf, E. R. Unanue, and B. Benacerraf. 1975. Binding of poly (Glu⁶⁰Ala³⁰Tyr¹⁰) by thymic lymphocytes from genetic responder and non responder mice: effect of antihistocompatibility serum. *J. Immunol.* 114:1670.
7. Shevach, E. M., W. E. Paul, and I. Green. 1973. Histocompatibility-linked immune response gene function in guinea pigs. Specific inhibition of antigen-induced lymphocyte proliferation by alloantisera. *J. Exp. Med.* 136:1207.
8. McDevitt, H. O., K. B. Bechtol, G. J. Hämmerling, P. Lonai, and T. L. Delovitch. 1974. Ir genes and antigen recognition. In *The Third ICN-UCLA Symposium on Molecular Biology*. E. E. Sercarz, A. R. Williamson, and C. F. Fox, editors. Academic Press, Inc., New York. 597.
9. Schwartz, R. H., C. S. David, D. H. Sachs, and W. E. Paul. 1976. T lymphocyte enriched murine peritoneal exudate cells. III. Inhibition of antigen-induced T lymphocyte proliferation with anti Ia sera. *Immunology.* 117:531.
10. Geczy, A. F., A. L. de Weck, B. D. Schwartz, and E. M. Shevach. 1975. Major histocompatibility complex of the guinea pig. I. Serologic and genetic studies. *J. Immunol.* 115:1704.
11. Eichmann, K., and K. Rajewsky. 1975. Induction of T and B cell immunity by anti-idiotypic antibody. *Eur. J. Immunol.* 5:661.
12. Binz, H., and H. Wigzell. 1975. Shared idiotypic determinants of B and T lymphocytes reactive against the same antigenic determinants. I. Demonstration of similar or identical idiotypes on IgG molecules and T-cell receptors with specificity for the same alloantigens. *J. Exp. Med.* 142:197.
13. Black, S. J., G. J. Hämmerling, C. Berek, K. Rajewsky, and K. Eichmann. 1976. Idiotypic analysis of lymphocytes in vitro. I. Specificity and heterogeneity of B and T lymphocytes reactive with anti-idiotypic antibody. *J. Exp. Med.* 143:846.

14. Eichmann, K. 1974. Idiotypic suppression. I. Influence of the dose and of the effector function of anti-idiotypic antibody on the production of an idio type. *Eur. J. Immunol.* 4:296.
15. Eichmann, K. 1975. Idiotypic suppression. II. Amplification of a suppressor T cell with anti-idiotypic activity. *Eur. J. Immunol.* 5:511.
16. Binz, H., and H. Wigzell. 1975. Shared idiotypic determinants on B and T lymphocytes reactive against the same antigenic determinants. II. Determination of frequency and characteristics of idiotypic T and B lymphocytes in normal rats using direct visualization. *J. Exp. Med.* 142:1218.
17. Prange, C. A., C. Green, D. E. Nitecke, and C. J. Bellone. 1977. Antigen binding lymphocytes in guinea pigs. I. B cell expansion to the monovalent antigen L-tyrosine-p-azophenyltrimethylammonium (tyr(TMA)) in the absence of antibody production. *J. Immunol.* 118:1311.
18. Bush, M. E., S. S. Alkan, D. E. Nitecki, and J. W. Goodman. 1972. Antigen recognition and the immune response. "Self-help" with symmetrical bifunctional antigen molecules. *J. Exp. Med.* 136:1478.
19. Alkan, S. S., D. E. Nitecki, and J. W. Goodman. 1971. Antigen recognition and the immune response. The capacity of L-tyrosine-azobenzene arsonate to serve as a carrier for a macromolecular hapten. *J. Immunol.* 107:353.
20. Inman, J. K., B. Merchant, L. Calfin, and S. E. Tacey. 1973. Coupling of large haptens to proteins and cell surfaces: preparation of stable, optimally sensitized erythrocytes for hapten-specific, hemolytic plaque assay. *Immunochemistry.* 10:1645.
21. Tabachnick, M., and H. Sobatka. 1960. Azoproteins. II. A spectrophotometric study of the coupling of diazotized arsanilis acid with proteins. *J. Biol. Chem.* 235:1051.
22. Porath, J., K. Aspberg, H. Drevin, and R. Axen. 1973. Preparation of cyanogen bromide-activated agarose gels. *J. Chromatogr.* 86:53.
23. Cheseboro, B., and H. Metzger. 1972. Affinity labeling of a phosphoryl-choline-binding mouse myeloma protein. *Biochemistry.* 11:766.
24. Gottlieb, A. B., R. K. Seide, and T. J. Kindt. 1975. Quantitative determination of allotypic and idiotypic markers with antisera coupled N-hydroxysuccinimide-activated sepharose. *J. Immunol.* 114:51.
25. Farah, F. S., M. Kern, and H. N. Eisen. 1960. The preparation and some properties of purified antibody specific for the 2,4-dinitrophenyl group. *J. Exp. Med.* 112:1195.
26. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.
27. Hansburg, D., D. E. Briles, and J. M. Davie. 1976. Analysis of the diversity of murine antibodies to Dextran B 1355 I. Generation of a large, pouciclonal response by a bacterial vaccine. *J. Immunol.* 117:569.
28. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of ¹³¹I-labelled human growth hormone of high specific radioactivity. *Biochem. J.* 89:114.
29. Davie, J. M., and W. E. Paul. 1971. Receptors on immunocompetent cells. II. Specificity and nature of receptor on dinitrophenylated guinea pig albumin. ¹²⁵I-binding lymphocytes of normal guinea pigs. *J. Exp. Med.* 134:495.
30. Hämmerling, G. J., and H. O. McDevitt. 1974. Antigen binding T and B lymphocytes. I. Differences in cellular specificity and influence of metabolic activity on interaction of antigen with T and B cells. *J. Immunol.* 112:1726.
31. Goodman, J. W., C. J. Bellone, D. Hanes, and D. E. Nitecki. 1974. Antigen structural requirements for lymphocyte triggering and cell cooperation. *Prog. Immunol.* 2:27.
32. Julius, M. H., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus derived murine lymphocytes. *Eur. J. Immunol.* 3:645.
33. Binz, H., and H. Wigzell. 1975. Shared idiotypic determinants on B and T lymphocytes reactive against the same antigenic determinants. IV. Isolation of two groups of

- naturally occurring idiotypic molecules with specific antigen-binding activity in the serum and urine of normal rats. *Scand. J. Immunol.* 4:591.
34. Hämmerling, G. J., and H. O. McDevitt. 1974. Antigen binding T and B lymphocytes. II. Studies on the inhibition of antigen binding to T and B cells by anti-immunoglobulin and anti-H-2 sera. *J. Immunol.* 112:1734.
 35. Geczy, A. F., C. L. Geczy, and A. L. de Weck. 1976. Histocompatibility antigens and genetic control of the immune response in guinea pigs. III. Specific inhibition of antigen-induced lymphocyte proliferation by strain-specific anti-idiotypic antibodies. *J. Exp. Med.* 144:226.
 36. Binz, H., and H. Wigzell. 1976. Shared idiotypic determinants on B and T lymphocytes reactive against the same antigenic determinants. V. Biochemical and serological characteristics of naturally occurring, soluble antigen-binding T-lymphocyte-derived molecules. *Scand. J. Immunol.* 5:559.
 37. Munro, A. J., and M. J. Taussig. 1975. Two genes in the major histocompatibility complex control immune response. *Nature (Lond.)* 256:103.
 38. Taniguchi, M., K. Hayakawa, and T. Tada. 1976. Properties of antigen-specific suppressive T cell factor in the regulation of antibody response of the mouse. II. *In vitro* activity and evidence for the I region gene product. *J. Immunol.* 116:542.
 39. Doherty, P. C., R. V. Blanden, and R. M. Zinkernagel. 1976. Specificity of virus-immune effector T cells for H-2K or H-2D compatible interactions: implications for H-antigen diversity. *Transplant. Rev.* 29:89.
 40. Shearer, G. M., T. G. Rehn, and A. M. Schmitt-Verhulst. 1976. Role of the murine major histocompatibility complex in the specificity of *in vitro* T-cell mediated lympholysis against chemically-modified autologous lymphocytes. *Transplant. Rev.* 29:222.
 41. Siskind, G. W., and B. Benacerraf. 1969. Cell selection by antigen in the immune response. *Adv. Immunol.* 10:1.
 42. Davie, J. M., and W. E. Paul. 1972. Receptors on immunocompetent cells. V. Cellular correlates of the "maturation" of the immune response. *J. Exp. Med.* 135:660.
 43. Moller, E., W. Bullock, and O. Makela. 1973. Affinity of T and B lymphocyte receptors for hapten determinants. *Eur. J. Immunol.* 3:172.
 44. Cohen, B. E., and W. E. Paul. 1974. Macrophage control of time dependent changes in antigen sensitivity of immune T lymphocyte populations. *J. Immunol.* 112:359.