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Immunology

T cells expressing the $V_\beta 1$ T-cell receptor are required for IgA production in the chicken

(IgA deficiency/V_β genes)

J. CIHAK*, G. HOFFMANN-FEZER†, H. W. L. ZIEGLER-HEIBROCK‡, H. STEIN*, B. KASPERS*, C. H. CHEN§, M. D. COOPER§¶, AND U. LÖSCH*‖

*Institute for Animal Physiology and ‡Institute for Immunology, University of Munich, and †Institute for Immunology, Gesellschaft für Strahlung and Umweltforschung, Munich, Federal Republic of Germany; and ¶Howard Hughes Medical Institute and §The Division of Developmental and Clinical Immunology, Departments of Medicine, Pediatrics and Microbiology, and the Comprehensive Cancer Center, University of Alabama, Birmingham, AL 35294

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ABSTRACT **While $\alpha\beta$ T cells in mammals may express one of many variable (V) gene families in the β locus, chickens have only two V_β gene families. The avian $V_\beta 2^+$ T cells are recognized by the T-cell receptor 3 (TCR3) monoclonal antibody and $V_\beta 1^+$ T cells are recognized by the TCR2 antibody, which we used to selectively suppress development of $V_\beta 1^+$ T cells in order to examine their functional role. Suppression was accomplished by multiple injections of anti-TCR2 antibodies beginning in embryonic life and perpetuated by thymectomy 8 days after hatching. Young birds thus depleted of $V_\beta 1^+$ T cells had greater than normal numbers of $V_\beta 2^+$ T cells and appeared as healthy as thymectomized and untreated controls. While production of IgM and IgG antibodies was unimpaired, IgA antibody production was severely compromised in the $V_\beta 1$-depleted birds. The levels of secretory IgA in bile and lung lavage fluid were reduced 1000- to 10,000-fold and secretory IgA antibodies were not produced in response to mucosal immunization. B-cell production of IgA antibodies thus appears to require T cells expressing the $V_\beta 1$ genes, whereas T cells that express the $V_\beta 2$ genes lack this capacity.**

Most of the T cells in mammals express an antigen receptor composed of α and β chains, while the others express a T-cell receptor (TCR) composed of γ and δ subunits (1). Avian homologues of the mammalian $\alpha\beta$ and $\gamma\delta$ TCRs can be identified with monoclonal antibodies. The chicken $\gamma\delta$TCR is identified with the TCR1 antibody (2) and two types of $\alpha\beta$TCR are identified in gallinaceous birds with the TCR2 and TCR3 antibodies (3–5). The key to this unexpected finding is provided by the recent cloning of genes in the chicken TCR β locus. This β locus contains two variable (V) gene families, one diversity (D) gene, four joining (J) genes, and a single constant region (C) gene (6). Chicken $\alpha\beta$ T cells therefore may express either $V_\beta 1$ or $V_\beta 2$ genes, the products of which can be recognized, respectively, by the TCR2 and TCR3 antibodies (7). While there is little sequence homology (30%) between the $V_\beta 1$ and $V_\beta 2$ genes, members within the two families are very similar (>95% homology). Thus, to an even greater extent than in mammals, which have many V_β gene families (1), TCRβ diversity in birds is generated primarily by junctional variations created by V–D–J_β splicing and insertion of nonencoded nucleotides (6). Interestingly, the numerous V_β genes in mammals can be assigned to two superfamilies on the basis of invariant sequences (8): $V_\beta 1$ superfamily gene members encode an arginine at position 64 and aspartic acid at position 86 to form a salt bridge between these residues, while $V_\beta 2$ genes instead feature an invariant codon for tyrosine at position 65. These features are retained in the chicken $V_\beta 1$ and $V_\beta 2$ genes, respectively (6).

The chicken therefore provides a relatively simple model for study of the functional roles of $\alpha\beta$ T cells by using the two prototype V_β genes. In earlier studies, we found that development of the TCR2 thymocyte population can be selectively inhibited by embryonic administration of the TCR2 ($V_\beta 1$ specific) monoclonal antibody (9, 10). When this antibody treatment is combined with surgical removal of the thymus after hatching, sustained suppression of the TCR2 cell population can be achieved in young birds. In the present study, we confirmed the selective suppression of $TCR2^+$ cells and observed a profound effect of this treatment on the integrity of mucosal immunity.

MATERIALS AND METHODS

Chickens. Chickens hatched from fertile outbred White Leghorn eggs obtained from LSL Vermehrungsbetriebe (Heinrichsruh, F.R.G.) were raised in our facility.

Antibodies. Murine monoclonal antibodies specific for chicken CD3 (11), TCR1 (2), TCR2 (3, 4), TCR3 (5), and an organophosphorus compound (control antibody F71H9) were produced in our laboratories. For purification of the TCR2 antibody, a protein G-Sepharose column (Pharmacia) was loaded with antibody-containing ascites in 20 mM phosphate buffer (pH 7.0), and the antibody was eluted with a glycine buffer (pH 2.7) dialyzed against phosphate-buffered saline (PBS), concentrated to 20 mg/ml, and filtered through a 0.2-μm filter. Monoclonal antibodies specific for chicken IgM, IgG, and IgA were provided by I. von Quistorp (M. Erhard and B. Kaspers, personal communication).

Embryonic Antibody Injections. Purified TCR2 antibody was injected intravenously (2 mg per embryo) on day 13 of incubation after cutting a window in the shell over a chorioallantoic vein and rendering the inner shell membrane translucent with a drop of sterile liquid paraffin (12). The injection with a 30-G needle was performed under a dissecting microscope and the window was sealed with transparent tape. A second injection of the antibody (5 mg per embryo) was given on day 17 of incubation. Three additional intravenous injections of the antibody (5 mg each) were given on days 1, 4, and 6 after hatching.

Thymectomy. Thymectomy was performed on the 8th day after hatching. The chicks were anesthetized by intramuscular injection of 0.1–0.15 ml of sodium pentobarbital solution (16 mg/ml), the neck region was entered via a dorsal incision, the thymus lobes contiguous to the carotid vessels were removed with forceps, and the wound was closed with metal surgical clamps.

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Abbreviations: TCR, T-cell receptor; V, variable; D, diversity; J, joining; MHC, major histocompatibility complex.

‖To whom reprint requests should be addressed at: Institut für Tierphysiologie, Veterinärstrasse 13, D-8000 Munich 22, F.R.G.

Collection of Bronchoalveolar Lavage Fluid, Bile, and Tears. Chickens were sacrificed by intravenous injection of pentobarbital. Flexible tubing was inserted into an incision of the trachea and 40 ml of saline was injected and retrieved with the syringe assisted by pressure on the thorax.

Bile was recovered after surgical exposure by direct puncture of the gallbladder. Tear fluid was obtained by using a Pasteur pipette from the conjuctival surface of living chickens.

Lymphocyte Isolation and Immunofluorescence Analysis. Lymphocytes isolated from whole blood samples by Ficoll/Hypaque centrifugation were examined for cell surface immunofluorescence. Viable cells (1×10^6) in 50 μl of PBS with 0.02% NaN_3 and 2% fetal calf serum (fluorescence buffer) were incubated for 30 min at 4°C with 50 μl of the CD3, TCR1, TCR2, or control antibody; washed three times; and then incubated with 50 μl of a 1:20 dilution of a fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Tago) for another 30 min at 4°C. After the second incubation, the cells were again washed three times, fixed with 1% paraformaldehyde, and analyzed by immunofluorescence flow cytometry using a FACScan Instrument (Becton Dickinson). Five thousand cells were analyzed per sample.

Enumeration of Circulating T Cells. Absolute counts of chicken blood leukocytes were performed as described (13). The absolute numbers of lymphocytes were calculated after performing a differential leukocyte count. The absolute numbers of T cells were estimated from the relative frequency of $CD3^+$ cells, and numbers of the TCR1, TCR2, and TCR3 cells were estimated from their relative frequencies as determined by FACS analysis.

Measurement of Immunoglobulin Levels. Immunoglobulin concentrations in serum and body fluids were measured by an ELISA using monoclonal antibodies specific for chicken IgG, IgM, and IgA. Polystyrene microplates (Nunc) coated overnight (4°C) with monoclonal antibodies specific for IgG, IgM, or IgA [2 μg/ml in PBS (pH 7.2); 200 μl per well] were then blocked with 0.5% gelatin in PBS (pH 7.2; 200 μl per well) for 60 min at 37°C. Serial dilutions of the samples were added to the plates (100 μl per well) and incubated at 37°C for 90 min. After washing three times with PBS, peroxidase-conjugated anti-IgG, -IgM, or -IgA monoclonal antibodies were added to the plates (100 μl per well) and incubated at 37°C for 90 min. The plates were then washed again and 100 μl of substrate, 2.5 mM orthophenyldiamine in 0.1 M citrate phosphate buffer (pH 5.0) with 0.015% H_2O_2 was added. After 10 min incubation the reaction was stopped with 2 M HCl (50 μl per well). Measurement was made at 492 nm with a Titertek photometer (Multiscan, Flow Laboratories). Concentrations of the three isotypes were estimated from a graph based on absorbances of the IgG, IgM, and IgA standards.

Determination of Anti-Tetanus IgA Antibodies. The IgA antibody response in the Harderian gland was examined after local immunization with tetanus vaccine (Behringwerke) according to a recently described method (14). Anti-tetanus antibodies of the IgA class were measured by ELISA. Briefly, microtiter plates were coated overnight at 4°C with tetanus toxoid (Behringwerke; 2 μg per ml) and then blocked with 4% skim milk powder solution in PBS for 1 hr. Serial dilutions (1:2) of tear samples from test chickens were added to the plates (100 μl per well) and incubated overnight at 4°C. The rest of the procedure was carried out as for ELISA estimates of IgA levels. IgA anti-tetanus antibody titers were expressed as the dilution at which the extinction was 0.1. When the 0.1 extinction was between two dilutions, the titer was calculated by interpolation to obtain a more precise estimate.

Immunohistochemistry. Spleen, bursa of Fabricius, ileum, lungs, cecal tonsils, and Harderian glands were removed from 6-week-old birds and snap frozen in liquid nitrogen. Cryostat sections (5 μm thick) were air dried and fixed with acetone for 10 min. The sections were incubated for 60 min with the mouse monoclonal antibodies or with normal mouse serum, washed with PBS and incubated for another 60 min with peroxidase-conjugated rat anti-mouse IgG (Jackson ImmunoResearch). Peroxidase activity was revealed with aminoethylcarbazole. The sections were counterstained with hematoxylin.

Statistics. Differences between sample values were evaluated by Student's t test. To analyze the relationship between the serum IgA level and the number of TCR2 cells, the correlation coefficient (r) was calculated.

RESULTS

Effect of Anti-TCR2 Treatment and Thymectomy on Development of TCR1, TCR2, and TCR3 Subpopulations. For *in vivo* depletion of the TCR2 population of cells, repeated intravenous injections of the monoclonal anti-TCR2 antibody were given before and after hatching. Between the 13th day of embryonic life and the 6th day after hatching, each bird received 22 mg of the anti-TCR2 antibody. The seeding of T cells expressing TCR1 or TCR3 was allowed to proceed normally until 8 days after hatching, when the animals were thymectomized in order to prevent further T-cell generation. The effect of this combined treatment on the development of T cells expressing the different TCR types was examined when the animals were 6 weeks old.

When the circulating pool of T cells was assessed by immunofluorescence analysis of peripheral blood mononuclear cells, it was found that thymectomy alone reduced the frequency of TCR1 cells, the relative frequency of TCR2 cells was increased, and the frequency of TCR3 cells was essentially unaltered (Table 1). Estimation of the absolute T-cell numbers in the blood indicated that thymectomy alone reduced the total T-cell population by almost 70%. The TCR1 subpopulation in the circulation was reduced by >90%, the TCR2 cells by 60%, and the TCR3 cells by >75% (Table 1). The effect of thymectomy alone on development of circulating TCR1 cells was the same as that of anti-TCR2 treatment plus thymectomy. However, the combined treatment resulted in selective suppression of the TCR2 cells (to <10% of control values) and a compensatory increase in TCR3 cells (>130% of controls).

The tissue distribution of T cells was assessed by immunohistochemical analysis of the spleen, intestine, cecal tonsils,

Table 1. Effect of the anti-TCR2 antibody treatment and thymectomy on T-cell development

Treatment	No. of animals	No. of cells per μl of blood (% of $CD3^+$ cells)			
		$CD3^+$	$TCR1^+$	$TCR2^+$	$TCR3^+$
Control	19	6520 ± 3239	1257 ± 493 (19)	4357 ± 2874 (67)	906 ± 446 (14)
Thymectomy	32	2070 ± 992	96 ± 86 (5)	1764 ± 811 (85)	210 ± 162 (10)
Anti-TCR2 and thymectomy	30	1666 ± 1121	97 ± 129 (6)	371 ± 354 (22)	1198 ± 811 (72)

Values are means ± SD from 6-week-old chickens. The frequency of $TCR3^+$ cells was calculated by subtraction of the frequencies of $TCR1^+$ and $TCR2^+$ cells from the total $CD3^+$ T-cell frequency since the anti-TCR3 antibody was not available throughout the study.

bursa, lungs, and Harderian glands. The analysis of the lymphoid tissues in normal controls confirmed the predominance of TCR2 over the TCR1 and TCR3 cells (Fig. 1 *b*, *d*, and *f*). While TCR3 cells were rarely seen in the small intestine, their relative frequency was higher in the spleen and cecal tonsils.

Thymectomy alone resulted in a profound decrease of the TCR1 cells in all tissues, except in the intestinal epithelium where the reduction was less prominent. This effect of thymectomy on the development of TCR1 cells was unaltered by the anti-TCR2 treatment. In contrast, while the number of TCR2 cells was only moderately reduced by thymectomy alone, the TCR2-expressing cells were reduced to 10–20% of control values in all lymphoid tissues examined in the anti-TCR2-treated and thymectomized birds (Fig. 1 *a* and *e*). In lymphoid tissues of the latter group of animals, the numbers of TCR3-positive cells were increased by as much as 5-fold (Fig. 1 *c* and *d*).

Effect of Anti-TCR2 Treatment and Thymectomy on Immunoglobulin Production. The TCR2-treated animals appeared to be as healthy as the controls suggesting that the TCR3 cells could compensate for the deficit in TCR2 cells. To explore this issue further, we measured serum immunoglobulin levels in the three groups of animals. While deficits were not observed in the IgM and IgG levels, the serum IgA levels were reduced to <10% of normal in the anti-TCR2-treated, thymectomized animals (Table 2). In chickens that were only thymectomized, the serum IgA levels were almost normal, and comparison of the numbers of TCR2 cells and the serum IgA levels indicated a positive correlation ($r = 0.81$; $P < 0.001$).

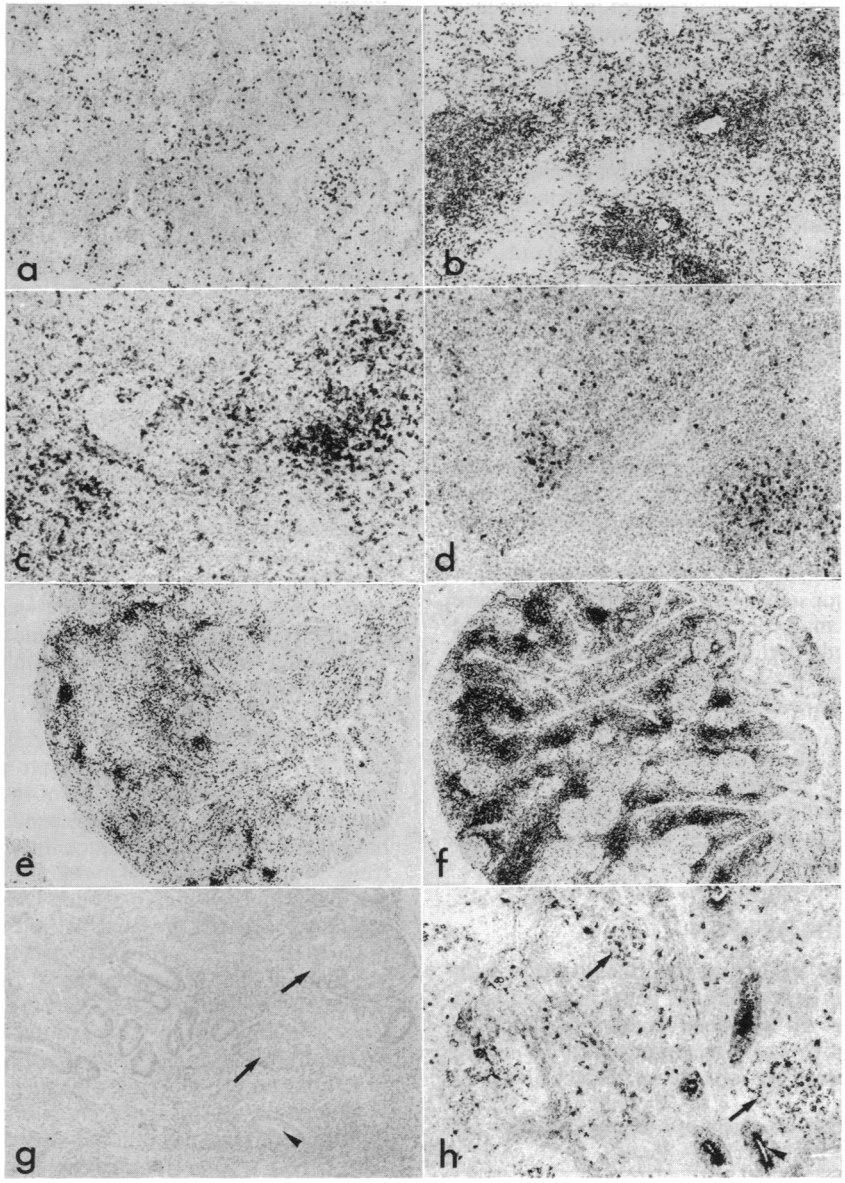

FIG. 1. (*a* and *b*) Distribution of TCR2-bearing lymphocytes in the spleen. Immunohistologic assessment of tissue sections reveals a profound reduction of the $TCR2^+$ cells in the spleen of an anti-TCR2-treated thymectomized chicken (*a*) and an abundance of $TCR2^+$ cells in the spleen of an untreated control animal (*b*). (×40.) (*c* and *d*) Demonstration of $TCR3^+$ lymphocytes in the spleen. The periarteriolar T-cell zones of an anti-TCR2-treated thymectomized chicken (*c*) contain increased proportions of $TCR3^+$ cells compared with the spleen of an untreated control chicken (*d*). (×70.) (*e* and *f*) $TCR2^+$ lymphocytes in the cecal tonsil. Cecal tonsils, like the spleen, reveal a striking reduction of $TCR2^+$ cells in a thymectomized anti-TCR2-treated chicken (*e*) and an abundance of $TCR2^+$ cells in an untreated control animal (*f*). (×20.) (*g* and *h*) Immunohistologic analysis of the distribution of intestinal IgA-containing cells. The cecal tonsil of an untreated control animal (*h*) contains many IgA^+ cells in the germinal centers (arrows). IgA accumulation is also seen at the apical surface of intestinal epithelial cells (arrowhead). The cecal tonsil of an anti-TCR2-treated, thymectomized chicken (*g*) lacks IgA-containing cells. (×40.)

Table 2. Effect of anti-TCR2 treatment and thymectomy on immunoglobulin production

Treatment group	No. of animals	Serum levels, μg/ml		
		IgG	IgM	IgA
Control	19	6268 ± 548	597 ± 80	302 ± 26
Thymectomy	32	5840 ± 686	786 ± 112	218 ± 22
Anti-TCR2 and thymectomy	30	5773 ± 646	868 ± 88	25 ± 7

*Values are means ± SE from 6-week-old chickens.

Because serum IgA levels may not correlate with those of secretory IgA in mucosal tissues, the levels of IgA were determined in bile and lung lavage fluids. As shown in Table 3, the animals treated with TCR2 antibody had levels of IgA in bile and lung lavage fluids that were 0.01–0.1% of the normal levels. Animals with the lowest serum IgA levels also had the lowest levels of secretory IgA. By contrast, chickens that were only subjected to thymectomy had modestly reduced levels of secretory IgA. The data indicate that TCR2-depleted animals are remarkably compromised in IgA production.

Tissue Distribution of Immunoglobulin-Producing Cells. When immunohistochemical analysis of the distribution of IgM-, IgG-, and IgA-producing cells in cecal tonsils, ileum, bursa of Fabricius, spleens, lungs, and Harderian glands was performed, IgA-containing lymphoblastoid and plasma cells were detected in all of these tissues of both control groups of animals. Bronchial and intestinal epithelial cells were also positive for IgA. In contrast, IgA-containing cells were not detected in the spleen, bursa, cecal tonsils, intestines, lungs, and Harderian glands of anti-TCR2-treated and thymectomized animals (Fig. 1 *g* and *h*). IgA was also not seen in epithelial cells of the mucosal surfaces in this experimental group of birds, while the numbers of IgM^+ and IgG^+ cells and their anatomical distribution were the same in experimental and control animals.

Evaluation of Local Secretory IgA Antibody Response. Specific IgA antibody responses in the Harderian gland were determined after intraocular stimulation with tetanus toxoid in two anti-TCR2-treated, thymectomized birds and in two normal control chickens. Anti-tetanus IgA antibodies could not be detected in the tears of the anti-TCR2-treated birds, whereas IgA anti-tetanus antibodies were easily detected in the tears of immunized controls (titers of 1:376 and 1:3500). IgA-producing cells were abundant in sections of the Harderian gland of the immunized controls, but no IgA-containing cells could be found even after local immunization of the anti-TCR2, thymectomized chickens.

DISCUSSION

The selective impairment of IgA production in birds with limited numbers of T cells expressing $V_\beta 1$ genes is a surprising and informative finding. In previous studies of chickens depleted of $V_\beta 1^+$ T cells by early treatment with the TCR2 antibody and thymectomy, we observed normal antibody responses after intravenous immunizations with sheep erythrocytes (ref. 8; unpublished observations). The normal IgM and IgG levels observed in the present experiments also indicate integrity of T cell help for production of antibodies of these isotypes. The apparent good health of the $V_\beta 1$-deficient, thymectomized group of birds further suggests overall immunological integrity. A significant role for the γδ T cells in IgA production is unlikely, since IgA production was severely compromised only in the $V_\beta 1$-deficient birds although the TCR1 population was reduced to the same extent in both the anti-$V_\beta 1$-treated, thymectomized birds and the thymectomized controls. The direct correlation observed between the numbers of TCR $V_\beta 1^+$ T cells and IgA production leads to the conclusion that the $V_\beta 1^+$ T cells are required for IgA antibody production in the chicken.

Table 3. Effect of anti-TCR2 treatment and thymectomy on IgA production

Treatment group	No. of animals	IgA levels, μg/ml		
		Serum	Lung lavage fluid	Bile
Control	7	385 ± 48	10 ± 3	16,446 ± 4327
Thymectomy	10	266 ± 81	6 ± 3	12,979 ± 6959
Anti-TCR2 and thymectomy	7	25 ± 4	0.01 ± 0.003	2 ± 2

*Values are means ± SE from 6-week-old chickens.

IgA production in mucosal tissues was especially compromised in birds deficient in $V_\beta 1^+$ T cells. While their serum IgA levels were reduced by ≈10-fold, IgA concentrations in bile and lung lavage fluids were decreased by 1000- to 10,000-fold. An attempt to elicit a mucosal IgA response by intraocular immunization with tetanus toxoid was completely unsuccessful in birds depleted of $V_\beta 1^+$ T cells. In keeping with these functional deficits, IgA-producing cells were virtually undetectable in sections of the small intestine, cecal tonsils, bursa of Fabricius, lung, Harderian ocular gland, and spleen of the anti-$V_\beta 1$-treated, thymectomized animals, while IgA-containing cells were abundant in the untreated and thymectomized controls. Overt clinical consequences of this breach in mucosal immunity in $V_\beta 1$-deficient birds will likely become evident in studies involving a longer period of observation and deliberate exposure to respiratory and intestinal pathogens.

The B-cell precursors of IgA-producing plasma cells are derived from IgM-bearing B cells generated in the bursa of Fabricius (15). The peripheral seeding of B cells capable of IgA production, with T-cell help (16), begins around the time of hatching (15). It is also known that B-cell production in the bursa is unimpaired by removal of the thymus (17). Normal development of the IgA B-cell precursors can thus be reasonably assumed in the birds that were depleted of $V_\beta 1$ T cells by anti-TCR2 treatment and thymectomy.

Our data thus raise the question of why the $V_\beta 2$ T cells, which were present in even higher than normal numbers in the $V_\beta 1$ T-cell-depleted birds, do not provide help for the differentiation of IgA-producing cells. One possibility is that the $V_\beta 2$ T cells lack the capacity to produce factors that are essential for the differentiation of IgA-producing cells. Interleukin 4 and other interleukin products of activated T cells have been shown to enhance IgA responses in mammals (refs. 14 and 18; reviewed in ref. 19). More information about the avian interleukins is needed to evaluate this possibility, but an attractive alternative is that most IgA responses are induced within the mucosal tissues and $V_\beta 2$ T cells simply do not migrate and proliferate there. In support of this idea, it has been shown that $TCR3^+$ cells are rarely found in the small intestinal lamina propria where the $V_\beta 1$ T cells are normally abundant (5), and this preferential homing pattern was confirmed in the present experiments.

Differences in the specificities of the $V_\beta 1^+$ and $V_\beta 2^+$ TCR for antigenic peptides and major histocompatibility complex (MHC) restriction elements also deserve consideration in the interpretation of our results. The CDR3 region formed by the VDJ_β junction is thought to interact directly with antigenic peptides held in the α-helical grooves of class I and class II molecules on antigen-presenting cells (1). Since the $V_\beta 1$ locus in the chicken contains a single D gene and the four J_β genes can be used in both $V_\beta 1$ and $V_\beta 2$ gene rearrangements (6, 7), the random addition of N sequences and variability in VDJ_β splicing should allow for the same range of CDR3 variability for both $V_\beta 1$ and $V_\beta 2$ TCRs. On the other hand, the fact that

the $V_\beta 1$ and $V_\beta 2$ genes share very little homology implies that these may interact differently with MHC molecules. In addition, β chains containing $V_\beta 1$ and $V_\beta 2$ sequences could exhibit differential pairing with α chains (5) containing various V_α sequences, the genes for which have not yet been cloned in the chicken. It is therefore possible that the $V_\beta 1$ and $V_\beta 2$ TCRs preferentially interact with different MHC restriction elements to dictate the $V_\beta 2$ T-cell responses to antigens entering the mucosal surfaces of the body.

These observations in the chicken may well have relevance for mammals. Mammalian T cells are known to be required for IgA production. Rabbits, mice, and humans depleted in T cells are especially compromised in the ability to produce antibodies of IgA isotype (20–23). Data obtained in mice have suggested the existence of a T cell capable of specifically inducing IgA isotype switching (24). Intestinal lymphoid tissues have served as a prominent source for clones of these so-called Tα cells (25, 26). It will thus be of interest to determine the relative homing characteristics and functional capabilities of mammalian T cells expressing the $V_\beta 1$ and $V_\beta 2$ gene superfamilies.

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