Direct demonstration of murine thymus-dependent cell surface endogenous immunoglobulin

(lymphoma cell/bone-marrow-derived lymphocyte/chicken antibody/κ chain/murine immunoglobulin fragments)

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ABSTRACT   Antisera raised in mammals to murine immunoglobulin (Ig) do not detect surface Ig on thymus-dependent (T) lymphoma cells as assessed by immunofluorescence analysis. In contrast, chicken antibodies, produced against the (Fab)$_2$ fragment of normal mouse IgG and purified by binding to and elution from IgG-Sepharose 4B, give strong indirect fluorescence with murine T cells and cultured T lymphoma cells. The surface Ig caps, is shed, and reappears, indicating that it is of endogenous origin. Nonlymphoid tumor cells of various myeloid types do not bind this reagent, even though they bear avid F$_c$ receptors. The capacity of chicken antibodies to bind to both bone-marrow-dependent and T cell lymphomas was abolished by adsorption with myeloma-derived κ chains coupled to Sepharose. The κ antigenic determinant recognized by the chicken antibodies may thus be different from that seen by mammalian antibodies, and the degree of exposure of the Ig on the T lymphoma surface might also affect ease of detectability with these reagents. These data provide direct evidence that T lymphocytes and T lymphoma cells express and synthesize a surface Ig containing determinants that at least crossreact with bone-marrow-cell-derived κ chains.

Murine thymus-dependent (T) cells generally do not bear immunoglobulin (Ig) detectable by immunofluorescence analysis using antisera raised in mammals (1, 2). This observation is somewhat paradoxical, however, because Ig has been isolated from solubilized T-cell plasma membranes by using such reagents (2–6). Several authors including Ramseier (7) and Binz et al. (8, 9) have reported recently that the idiotypes of circulating anti-allotypes or anti-strep. A antibodies correspond to the idiotypes of antigen receptors on T cells sensitized to the same antigens. It has also been reported recently by Hammerling et al. (10) that some antibodies produced in fowls to MOPC 104E (λμ) myeloma protein detected an Ig on mouse T cells. Chicken antisera specific for human Fab were shown to bind to human T cells (11). In the present paper we endeavored to develop anti-immunoglobulin reagents that would visualize T-cell Ig by immunofluorescence. Because mammalian antisera readily detect surface Ig on virtually all lymphocytes, including thymocytes of many lower species (12, 13), phylogenetic distance appears to be an important factor in the generation of antibodies reactive with T cell Ig. We chose chickens as our source of antibody because birds are thought to represent an offshoot of dinosaurs (14) that diverged early from the reptilian line leading to mammals. To increase the likelihood that the antibodies raised in this species would react with variable region determinants, the immunogen used was the (Fab)$_2$ fragment of IgG immunoglobulin isolated from normal mouse serum.

Abbreviations: T cell, thymus-derived cell; FCS, fetal calf serum; B cell, bone-marrow-derived cell.
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We report that avian antibodies raised in this manner and purified by binding to and elution from solid-phase immunoadsorbents give strong indirect immunofluorescence with murine thymus cells, peripheral T cells, and T lymphoma cells.

MATERIALS AND METHODS

Animals and antigens

Fowls. Fertile eggs (outbred White Leghorn Australorps hybrids) were obtained commercially. Chickens were hatched and reared in our animal house. Immunization was done at the age of 3–4 months.

Mice. CBA, C3H, C57Bl, and CBA-nu/nu were obtained from our animal house and used at the age of 2–3 months.

Rats, Rabbits, and Guinea Pigs. These animals were all bred in our animal house.

Mouse IgG and (Fab)$_2$. Mouse IgG was prepared from normal serum by zone electrophoresis on starch followed by gel filtration on Sephadex G-200. The preparation was further purified by binding to and elution from *Staphylococcus aureus* protein A coupled to Sepharose (Pharmacia, Uppsala). IgG was digested with pepsin as previously described (15). Intact IgG and F$_c$ fragments were removed by binding to protein A-Sepharose (16).

Cell suspensions

Thymus or spleens were gently pressed through fine stainless steel mesh into HEM, and spun down; then red cells and damaged cells were removed (17). Mouse tumor cells are continuously cultured *in vitro* in the Institute and were generously provided by A. Harris.

Immunization and isolation of antibody

Fowls were immunized by injection of mouse (Fab)$_2$ (2 mg per fowl) in complete Freund's adjuvant into both leg and breast muscles. The first bleed took place 3 weeks later. Fowls were boosted 3–4 weeks later in the same way and bled 2 weeks later. Immune sera were adsorbed on a column of mouse IgG coupled to Sepharose (5 mg of IgG per ml of gel) and specific antibodies were eluted with glycine·HCl buffer, pH 2.5, which was 150 mM in NaCl. After dialysis against phosphate-buffered saline, the protein concentration was adjusted to approximately 0.6 mg/ml. The purified antibody consisted predominantly of 7S IgG (IgY) as assessed by polyacrylamide gel electrophoresis. Purified antibody from fowl 5399 (first bleed) was used for all the experiments reported on in this paper. Similar results were obtained with later bleeds and antibodies from other chickens immunized in this manner.

Control sera

To avoid "natural antibodies" to mouse tissue present in normal adult fowl, we obtained serum-control serum from newly

hatched chickens, and Ig fraction was prepared by Na_2SO_4 precipitation at room temperature. For use, protein concentration was adjusted to 0.6 mg/ml. To avoid the possibility that "natural antibodies" might still be present in the purified fowl anti-mouse $(Fab)_2$ antibody preparation, antibodies prepared in fowls to human and rabbit $(Fab)_2$ and purified on solid phase immunoadsorbents were used as additional controls.

Absorption of antisera

Mouse κ chains were conjugated to Sepharose by cyanogen bromide. The κ chains were prepared by agar block electrophoresis and gel filtration from serum of mice bearing plasmacytoma HPC-114. This tumor secretes κ light chains as disulfide-linked dimers. Analysis of the separated chains on polyacrylamide gels showed this preparation to contain only Ig light chains. Mouse Ig column was the same as described above.

Fowl antibodies specific for mouse $(Fab)_2$ were absorbed by passage through one or the other column. Nonconjugated Sepharose column was used as a control for nonspecific absorption and to calculate the dilution factor.

Staining procedures

Cells, 2 to 4 × 10^6 cells per plastic Wasserman tube, were resuspended in 0.15 ml of Eisen's balanced salt solution, and 10 μg of specific antibody or control Ig preparation was added. Tubes were incubated for 40 min on ice, then the suspension was layered on top of a step-wise fetal calf serum (FCS) gradient (100%, 75%, 50%, 25%, 0% FCS in Eisen's solution) and spun at 400 × g for 5 min. The pellet was resuspended in 0.15 ml of Eisen's solution, and fluorescein-conjugated rabbit IgG anti-fowl Ig (fluorescein/protein ratio 1.5–2.5) was added, as approximately 30 μg of protein per tube. After another 40 min incubation on ice, the cells were again washed through an FCS gradient, and the pellet was dispersed in a few drops (according to cell number) of Eisen's. The suspensions were examined with a Leitz Orthoplan microscope with Ploem epi-illuminator and HBO 200 light source under ×100 oil immersion objectives and ×10 periplan oculars.

Capping and resynthesis experiments

Cells were treated exactly as above, only all the incubation took place at room temperature. After the capping with fluorescein-conjugated rabbit anti-fowl Ig, one lot of cells was treated immediately with fowl anti-mouse $(Fab)_2$, incubated on ice for 40 min, and then treated with rabbit IgG anti-fowl Ig conjugated to Rhodamine (R-P ratio 3.5), a gift from J. Goding, for another 40 min. The second lot was incubated in DMS culture medium for 4 hr at 39°‡ with 10% CO_2 in air. After the incubation the cells were spun, resuspended, and treated as described under *Staining procedures* by using Rhodamine-conjugated rabbit antibody in *Cell suspensions* procedures.

Autoradiography

Cell suspensions (2 to 4 × 10^6 in 0.15 ml) were incubated on ice for 40 min with [125I]-conjugated fowl anti-mouse Fab_2 antibody preparation, or fowl anti-human $(Fab)_2$, approximately 15 μg, 35 μCi per tube, washed twice through an FCS gradient, resuspended in a few drops of FCS, and smeared on gelatinized microscope slides. The slides were dried quickly with a hair drier, and then fixed for 5 min in methyl alcohol. The slides were dipped in Kodak NTB emulsion, exposed for 4 days, and

‡ Incubation at 39°, instead of the usual 37°, did not serve any specific purpose. The incubator in the author's (A.S.) laboratory, mostly used for culture of chicken lymphocytes, was set for this temperature.
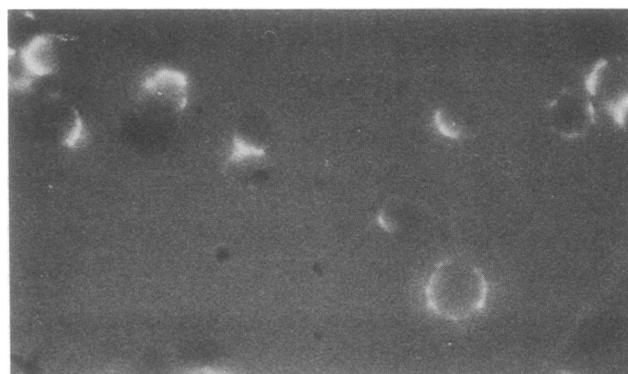
FIG. 1. Fluorescent CBA thymocytes after treatment with fowl anti-mouse $(Fab)_2$ antibodies and fluorescent rabbit IgG anti-fowl Ig.

then developed and stained with methyl green-pyronin. Two hundred cells were counted per slide.

Surface radioiodination and isolation of immunoglobulin

Thymus lymphocytes of CBA mice and spleen cells of CBA-nu/nu mice were prepared (17) and labeled with [125I] iodide in a lactoperoxidase-catalyzed reaction as described (18). Labeled cells were disrupted either with 10 M urea/1.7 M acetic acid/0.1% Triton X-100 (3) or 1% Triton X-100 (19) in phosphate-buffered saline, pH 7.3. Immunoglobulin was isolated by using a "sandwich" coprecipitation system in which 200 μl of cell lysate was incubated either with 5 μg of chicken antibody to mouse $(Fab)_2$ (specific) or 5 μl of chicken IgG that had been passed through mouse IgG-Sepharose to remove antibodies to mouse immunoglobulin (control). Precipitates were developed by the addition of 50 μl of rabbit antiserum to chicken immunoglobulin. Precipitates were washed, solubilized, and subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate as described (20).

RESULTS

Ninety percent of mouse thymocytes from the three strains tested (CBA, C57B1, and C3H) showed strong fluorescence after treatment with purified fowl anti-mouse $(Fab)_2$ antibody and rabbit IgG anti-fowl Ig fluorescent reagent (Fig. 1). Suspensions treated with normal neonatal fowl Ig or fowl anti-human $(Fab)_2$ were uniformly negative. Fluorescein-conjugated rabbit IgG anti-fowl Ig, used alone, stained only dead cells. Normal spleen cells and spleen cells from CBA-nu/nu mice were also approximately 90% positive, which indicates that bone-marrow-derived (B) cells and peripheral T cells react with this antibody preparation.

To obtain more quantitative data on the percentage of positive cells and relative number of Ig molecules on the surface of both thymocytes and B cells, autoradiographic slides were examined and grain count distribution was assessed. As can be seen from Fig. 2, 93% of thymocytes and 89% of nude spleen cells were positive. These numbers will approximately correspond to the proportion of lymphoid cells in our preparations. Not unexpectedly, B cells seem to bind approximately five times more antibody molecules than T lymphocytes.

Tumor Cell Lines. The results are shown in Table 1. All the lymphoid lines with the exception of EL-4 thymoma showed positive fluorescence. The intensity of fluorescence varied considerably between the tumor lines. Nonlymphoid tumor lines, known to possess avid F_c receptors, such as WEHI-265 myeloid leukemia and P815 mastocytoma, did not show any fluorescence.
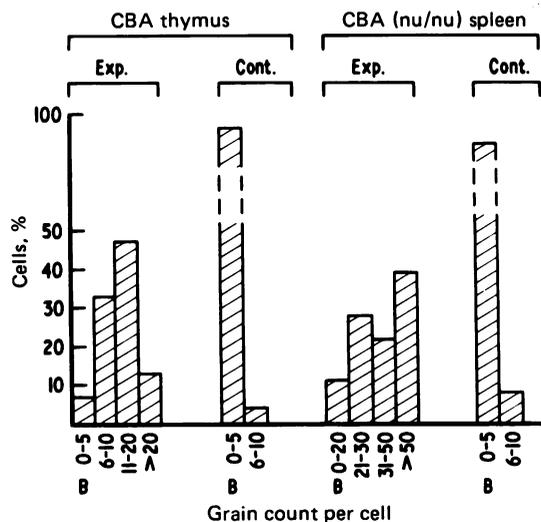
FIG. 2. Grain count distribution in preparation of CBA thymocytes and CBA-nu/nu spleen cells. Letter B indicates the level of background grain counts over an area corresponding to single cells. Exp., experimental; cont., control.

Good crossreactivity was observed with rat and guinea pig thymocytes, which gave nearly as strong fluorescence as mouse cells.

Rabbit thymocytes and blood lymphocytes were very weakly fluorescent; human blood lymphocytes were completely negative.

Capping and resynthesis experiments

Thymocytes treated with specific antibody and fluorescein-conjugated rabbit IgG anti-fowl Ig at room temperature showed uniformly (over 80%) green caps. If treated immediately afterwards with specific antibody and Rhodamine-conjugated rabbit IgG anti-fowl Ig, the caps showed both green and red fluorescence, but uniform cell surface fluorescence was not observed. Cells capped as above, incubated for 4 hr under tissue culture conditions, and then treated with specific antibody and Rhodamine-conjugated rabbit anti-fowl Ig also showed green and red caps, the green caps being frequently incorporated already into the cytoplasm. In addition, distinct red-ring fluo-

rescence was observed, thereby indicating the reappearance of surface immunoglobulin.

In the second experiment, the cells were capped off as in the previous experiment, and then incubated under tissue culture conditions up to 15 hr. At indicated time points, aliquots were washed through the FCS gradient and restained on ice with fowl anti-mouse $(Fab)_2$-specific antibodies and rabbit IgG anti-fowl Ig fluorescent preparation. Two hundred cells were counted per preparation. Table 2 shows that nearly complete regeneration of the surface "Ig" of thymocytes takes place after 6 hr of culture and is complete after 15 hr.

Specificity of Fowl Anti-Mouse (Fab)₂ Antibodies. The results in Table 3 demonstrate that passage of the fowl antibody preparation over κ chain-Sepharose conjugate completely removed all binding activity to murine B and T lymphomas and to normal CBA thymocytes. Absorption on Sepharose column did not remove the binding activity. The B lymphomas express IgM (WEHI 279) or IgG₂ (2PK-3). These data indicate that the fluorescence activity is due solely to an antibody directed against antigenic determinants on mouse κ chains.

Table 1. Strength of fluorescence of different cells relative to CBA thymocytes

	Fluorescence
CBA thymocytes	++
CBA-nu/nu splenocytes	+++
S49 thymoma	++
WEHI-231 B lymphoma	+++
WEHI-7.1 T lymphoma	++
RILQ T lymphoma	+
WEHI-22 T lymphoma	+++
WEHI-112 T lymphoma	+
WEHI-265 myeloid leukemia	−
EL 4 thymoma	−
P815 mastocytoma	−
Rat thymocytes	++
Guinea pig thymocytes	++
Rabbit thymocytes	±
Human peripheral blood lymphocytes	−

Fluorescence of CBA thymocytes was arbitrarily classed as ++. ±, very weak fluorescence; +, weak fluorescence; ++, strong fluorescence; +++, very strong fluorescence.

Table 2. Kinetics of the regeneration of "Ig" on the surface of thymocytes after capping off

	% cells					
		Length of incubation, hr				
	Starting population	1	2	4	6	15
No fluorescence	8	9	35	11	12	10
Strong ring fluorescence	75	0	0	3	44	69
Weak ring fluorescence	17	0	0	61	41	21
Patches	0	62	0	0	0	0
Caps	0	29	42	9	0	0
Interiorized caps	0	0	23	0	0	0
Caps + ring	0	0	0	8	3	0

Table 3. Absorption of anti-immunoglobulin staining of T- and B-cell lymphomas by κ-Sepharose conjugate

		% fluorescent cells*	
Tumor line	Type	Chicken anti-mouse Fab	Chicken anti-mouse Fab absorbed HPC-114 Sepharose
WEHI-7.1	T lymphoma	32	0.5
S49.1	T lymphoma	87	2.2
WEHI-279	B lymphoma	96	1.3
2PK-3	B lymphoma	91.5	0.5
CBA	Thymus lymphocytes	92.0	4.0

* Chicken antibody followed by fluorescein-conjugated rabbit anti-chicken Ig.

Absorption of the antibodies on mouse $(Fab)_2$ column produced identical results. Absorption on M315 (α,λ) myeloma protein column did not reduce the binding activity.

Protein estimation before and after absorption showed that κ chains removed 80% of protein, $(Fab)_2$ removed 75%, and Sepharose alone removed approximately 5%.

Radioimmunoassay analysis of the specificity of these antibody preparations showed that the majority of the activity is directed against a κ chain determinant expressed on all classes of Ig molecules.

Isolation of surface immunoglobulins of thymus and spleen lymphocytes

Approximately 1% of high molecular weight ^{125}I-labeled protein of CBA thymus cells or CBA-nu/nu spleen cells was specifically precipitated whether the disruption medium was 0.1% Triton X-100 or 0.1% Triton X-100 in acid urea. The solubilized precipitates were analyzed by polyacrylamide gel electrophoresis (10% acrylamide) under reducing conditions as illustrated in Fig. 3. The nu/nu spleen cells gave a pattern typical of murine B cells in which light chains and μ and δ-like heavy chains were resolved. The specific precipitate of thymus immunoglobulin contained light chains and a major heavy chain peak migrating between the positions of μ and δ. A small component with a relative mobility of approximately 0.53 was observed as well as a larger component in the relative mobility range of 0.07–0.09. By comparison with nonimmunoglobulin standards (21), the major heavy chain has a nominal mass of 68,000 daltons. The higher-molecular-weight component has a mass of about 140,000 by comparison with IgG and μ chain standards. The amount of this component varied from experiment to experiment.

DISCUSSION

These data show that chicken antibodies having specificity for murine κ chains enable the autoradiographic and immunofluorescent demonstration of surface immunoglobulin on thymus cells and monoclonal T lymphoma cells. Although we have not yet established all the reactivities of the chicken anti-Fab antibodies, the anti-κ activity is the relevant one in the present study. The immunoglobulin is of endogenous origin as indicated by capping and resynthesis experiments and from the use of cultured cloned tumor cell lines.

Surface Ig isolated from T and B cells by using chicken antibodies was compared to that previously described by using either mammalian (4) or avian antiserum (22). Our results support previous findings that the heavy chain of T cell Ig can be distinguished electrophoretically from serum or B cell μ chain (4, 5, 23). We believe that the component of nominal molecular weight 140,000 is probably a dimer of heavy chains which has failed to dissociate. Hammerling *et al.* (22) provided evidence that murine T-cell Ig precipitated by avian antibody has a pronounced tendency to aggregate and strong dissociating conditions are required to prepare monomeric light and heavy chains.

The present results offer strong evidence for the presence of endogenously produced κ chains on murine T cells. Comparison of these data with results obtained by using sheep antiserum to mouse κ chain raises fundamental questions regarding the nature or display of T cell-surface Ig. The sheep antiserum stains B cells extremely well, but shows no reactivity for T cells. It is possible that the sheep antiserum recognizes different determinants than does chicken antiserum. If, for example, T cells and B cells possess the same κ chains this dichotomy might be explained by the chicken antiserum being directed towards
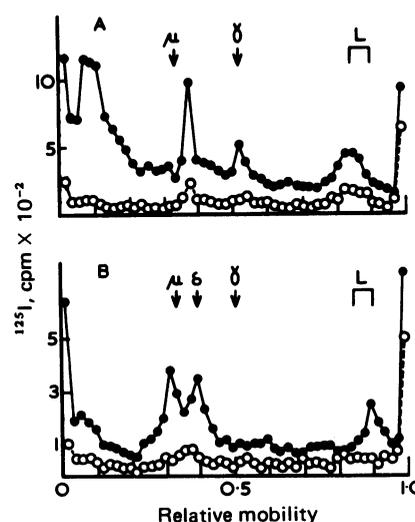
FIG. 3. Analysis of mouse lymphocyte surface Ig on sodium dodecyl sulfate polyacrylamide gels. (*A*) Surface Ig of CBA thymocytes extracted with acid urea/0.1% Triton X-100. (*B*) Surface Ig of CBA-nu/nu spleen cells extracted with 0.1% Triton X-100. ●, specific anti-immunoglobulin precipitate; O, control precipitate. See *Materials and Methods* for definitions.

V-region determinants that are exposed and the sheep antiserum reacting only with C-region determinants that are buried. An alternate explanation is that T cell light chain is coded for by a distinct light-chain gene that is related in its evolution to κ chain gene and the gene product, and possesses crossreacting determinants that are seen by avian but not by mammalian antisera. The results of Hammerling and his associates (10, 22) using avian antisera directed against murine μ chains parallel this situation. A remote possibility, which cannot be excluded on the basis of presently available data, would be the assumption that some ubiquitous antigen on the surface of lymphocytes (i.e., an MHC dependent marker) possesses sufficient areas of homology with κ chains to give serological crossreactivity. But positive evidence that such crossreactivity does exist seems not to be available. Further analysis is needed to determine whether the antigen determinant on the κ chain is located within the V or C region. Demonstration of endogenous light chains on T cells is not inconsistent with the concept of T-cell immunoglobulin existing as a dimer of heavy chains that was derived by using anti-idiotype antisera (8), because recent evidence indicates that T-cell light and heavy chains are not covalently linked (6, 19, 24), whereas the heavy chains can occur as a disulfide-bonded dimer (24).

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