IMMUNOCOMPETENT CELLS OF THE CHICKEN

I. Specific Surface Antigenic Markers on Bursa and Thymus Cells*

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(Received for publication 9 June 1971)

The bursa of Fabricius (1) in the chicken is generally considered to be a temporary site of concentration, possibly also of maturation, of bone marrow–derived (2) precursors of antibody-forming cells, "B-cells"[1] (3, 4). Conversely, thymus-derived or "T-cells" are responsible for such major phenomena as homograft rejection (3, 4) and delayed-type hypersensitivity (5). Experiments on neonatal removal of these two central lymphoid organs have clearly established these functions in the immune response. In this respect the thymus of the chicken appears to function similarly to that of the mammal, but evidence for cellular cooperation between B- and T-cells in the chicken analogous to that found in mammals (6, 7) is still lacking (8).

One way of studying the relative roles of cooperating cells in peripheral lymphoid organs is by means of cell fractionation methods or by selective killing of a given cell type, which can be accomplished by means of incubation of cells with complement and antisera to surface antigens. Analysis of the role of T-cells in the primary and secondary response to sheep erythrocytes (SE) in the mouse has been accomplished by preferential killing of the T-cells with alloantisera to θ-antigen (9). Several thymus-specific antigens such as θ (10, 11) have been found on mouse thymocytes (12, 13).

For the demonstration of lymphoid cell cooperation in the chicken it would be advantageous to have antisera which can specifically recognize and inactivate B- and T-cells. Since a variety of inbred lines of chickens are not readily available, the approach followed in mice of preparing alloantisera is impossible. In the present study, antisera to chicken bursa and thymus were prepared in rabbits and were found,

* Supported in part by U.S. Public Health Service grant AI-3076 and by grant No. T-524D from the American Cancer Society.

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[1] *Abbreviations used in this paper:* B-cells, bone marrow–derived precursors of antibody-forming cells; C′, complement; CBLA, chicken B-lymphocyte antigen; CBuLA, chicken bursa-derived lymphocyte antigen; CTLA, chicken T-lymphocyte antigen; DPFC, direct plaque-forming cells; IPFC, indirect plaque-forming cells; MBLA, mouse B-lymphocyte antigen; MSLA, mouse specific lymphocyte antigen; RPMI, Roswell Park Memorial Institute; SE, sheep erythrocytes; T-cells, thymus-derived cells.

after appropriate absorption, to react specifically with surface antigens on the cells from these two lymphoid organs.

Immunoglobulin constitutes another surface marker of murine lymphoid cells, which appears to be primarily on B-cells (14).[2] This property appears to be paralleled in the chicken, since the bursa is the only site of detectable immunoglobulin synthesis in the chick embryo (15, 16) and injection of antisera to chicken μ-chains into the embryo delays immunoglobulin synthesis (17). Therefore, studies were done on the ability of antisera to chicken immunoglobulin to kill, in the presence of C′, chicken lymphoid cells of varying origin, including antibody-forming cells. The results to be presented show that such antisera are cytotoxic for bursa but not for thymus cells.

Materials and Methods

Animals.—Outbred White Leghorn chickens 4 wk of age, obtained from Shamrock Poultry and Breeding Farm, North Brunswick, N.J., were normally used. In some experiments F_1 (strain No. 96) White Leghorn chickens, acquired as hatching eggs from Hy-Line Poultry Farms, Johnston, Iowa, were also included. The results of the experiments were not detectably influenced by the strain of the chickens. Use of chickens older than 4 wk was carefully avoided because previous studies on cell transfers (8) and histology of chicken lymphoid organs (18) suggested an increasing degree of mixing of different cell types in the central lymphoid organs with age.

Bursectomized, severely hypogammaglobulinemic chickens were obtained by injection of 3.5 mg testosterone propionate into 12-day-old embryos followed by 650 R X-irradiation on the day after hatching (4). Sera from these animals were screened by immunodiffusion against rabbit anti-chicken Ig for the absence of detectable immunoglobulins.

For the induction of antibody-forming cells adult chickens were injected intravenously with 0.2 ml 20% SE and their spleens removed 5–10 days later. Adult male New Zealand white rabbits were used for the preparation of antisera to chicken bursa and thymus cells and to chicken immunoglobulin.

Antisera and C′.—Antisera against bursa or thymus cells were elicited by two injections within a 2 wk interval of bursa or thymus cell suspensions (anti-thymus sera Nos. 1 and 3; anti-bursa sera Nos. 1 and 2) or by injection of a mixture of cells and complete Freund's adjuvant subcutaneously (anti-thymus sera Nos. 2 and 4; anti-bursa Nos. 3 and 4). The rabbits were bled several times between 7 and 21 days after the second injection. All bleedings from each individual rabbit were pooled. Small samples of each antiserum were heat-inactivated at 56°C for 30 min and absorbed extensively at 0°C with ½ ml packed chicken erythrocytes followed by ½ ml packed bursa or thymus cells/ml of antiserum. In some cases more than one absorption was needed. Control normal rabbit sera were heat-inactivated and absorbed similarly with chicken erythrocytes followed by a mixture of bursa, thymus, and spleen cells.

Anti-chicken L-chain serum was prepared in a rabbit by two injections, at a 4 wk interval, of 500 μg L-chains in complete Freund's adjuvant. The animals were bled repeatedly starting 1 wk after the last injection. The chicken L-chains used were obtained through the kindness of Dr. A. Benedict, Department of Microbiology, University of Hawaii, Honolulu. Anti-chicken

[2] Takahashi, T., L. J. Old, K. R. McIntyre, and E. A. Boyse. 1971. Immunoglobulin and other surface antigens of cells of the immune system. *J. Exp. Med.* **134**:815.

Ig was prepared in rabbits by two biweekly injections of 5 mg chicken γG, obtained by the method of Benedict (19), in complete Freund's adjuvant. The animals were bled repeatedly and sera were analyzed by double diffusion in agar and by immunoelectrophoresis. The specific antisera formed only one line with whole chicken serum. Both anti-L and anti-γG reacted with the L-chain preparation. Absorption of these antisera was accomplished with packed thymus cells from hypogammaglobulinemic chickens or in some cases with thymus cells from normal young chickens. Absorption with erythrocytes was omitted in order to avoid loss of anti-Ig activity.

Fresh guinea pig serum was used as the complement source. It was absorbed at 0°C with a mixture of bursa, thymus, and spleen cells, and with erythrocytes until acceptable cytotoxicity levels for thymus cells were reached (10% dead cells or less). The method of Boyse et al. (20) was followed to absorb sera for use as a C′ source, employing 0.01 M ethylenediaminetetraacetate (EDTA), pH 7.0, to prevent loss of C′ activity.

Cytotoxicity Test.—Cell suspensions were obtained by extremely gentle teasing of slices from each organ into medium determined to be optimal for that tissue's cell viability. Thymus was teased into Dulbecco's modified Eagle's medium,[3] spleen into RPMI 1640[3] containing 10% normal chicken serum or fetal calf serum,[3] and bursa into RPMI 1640 containing 10% fetal calf serum and ⅛% gelatin.[3] Cells were washed and collected by centrifugation. The cytotoxicity test was performed by adding to 0.1 ml samples of serial twofold dilutions of the test antiserum the following: 0.05 ml of C′, 5×10^6 thymus, bursa, or spleen cells, and the proper medium to make the final volume in the tube 0.5 ml.[4]

The tubes were incubated for 40 min at 37°C in an atmosphere of 5% CO_2 and air. For testing the effect on plaque-forming cells, immune spleen cells were similarly incubated with the antisera at a 1:20 dilution. The degree of cytotoxicity was assayed by the trypan blue exclusion method (21).

Assay of Plaque-Forming Cells.—Immunized spleens were assayed for both direct and indirect anti-SE plaque-forming cells by the method of Jerne et al. (22). Direct plaques were developed by 1:6 dilution of fresh chicken serum as the source of C′. Indirect plaques were developed by addition of polyvalent rabbit antiserum to chicken Ig followed by guinea pig C′ (23).

RESULTS

Specificity of Antisera.—

Anti-thymus sera: All four anti-thymus sera, after extensive absorption with bursa cells, were cytotoxic in the presence of guinea pig C′ for >90% of thymus cells at a final dilution of 1:160 or less (Fig. 1). Absorbed normal rabbit serum in the presence of C′ never killed more than 10% of thymus cells. The similarity in the relative activities of the four antisera against thymus cells was striking.

The cytotoxicity was always much less for bursa than for thymus cells. At dilutions higher than 1:20 these anti-thymus sera had very little cytotoxic activity for bursa cells above the background obtained with similar dilutions of absorbed normal rabbit serum (up to 12%). At the lowest dilution tested (1:10)

[3] All media were obtained from Associated Biomedic Systems, Inc., Buffalo, N.Y. K and K Gelatin No. 1592 was generously donated by the Knox Gelatine Co., Johnstown, N.Y.

[4] This order of additions was strictly adhered to in view of the findings of Takahashi et al.[2] with anti-Ig sera in the murine system.

the antisera killed a variable percentage of bursa cells ranging from 4 to 21% above background (Fig. 1).

It should be noted that unabsorbed rabbit sera are also much more toxic for thymus than for bursa cells. Although much more variable than the absorbed anti-thymus sera, some normal sera were tested that in dilutions of $\leqq 1:40$ killed >90% thymus cells. This activity was, of course, always removed from the control sera by absorption with thymus

Anti-bursa sera: Two of the four anti-bursa sera tested were no longer significantly cytotoxic for thymus cells at dilutions 1:20, while they killed >90% of bursa cells in dilutions up to 1:160 (Fig. 2, sera Nos. 2 and 4). At the lowest dilution tested (1:10) these antisera killed 0 and 6% of thymus cells above background. The other two antisera (Fig. 2, sera Nos. 1 and 3) were somewhat less active against bursa cells, although they were also much more cytotoxic for bursa than for thymus cells.

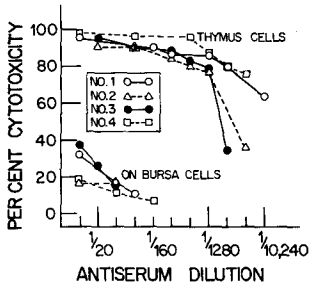

FIG. 1. Degree of cytotoxicity of various dilutions of different absorbed rabbit anti-thymus sera on chicken thymus and bursa cells.

Anti-immunoglobulin sera: Absorption of these antisera with thymus cells removed cytotoxicity for thymus (Fig. 3), whereas anti-immunoglobulin activity was not affected by such absorption. These absorbed antisera at a dilution of 1:80 still killed 70 or 80% of bursa cells.

While addition of chicken serum did not inhibit the cytotoxicity of anti-bursa and anti-thymus sera for bursa and thymus cells, respectively, addition of 0.05 ml of chicken serum to a typical incubation mixture for the cytotoxicity test reduced the killing effect of both anti-immunoglobulin sera on bursa cells to background level.

Effect of antisera on spleen cells: In agreement with the findings of others (23), it was found that during the response of the chicken after an intravenous injection of SE, indirect (IPFC) and direct plaque-forming cells (DPFC) appear simultaneously. For example, on day 5 about 1000 DPFC and 9000 IPFC/10^6 spleen cells were detected. Since the numbers of IPFC were always much higher than those for DPFC no effort was made to subtract the DPFC from the IPFC, even though DPFC were probably detected by both methods.

Incubation of the SE-immune spleen cells with anti-bursa sera at a dilution of 1:20 and C′ greatly reduced the number of IPFC, and caused a smaller but significant reduction of DPFC (Table I). The percentage of spleen cells killed by these antisera was relatively low, and could not be adequately evaluated, since it was not always possible to predictably keep the background level of the cytotoxicity test with spleen cells after incubation with normal rabbit serum and

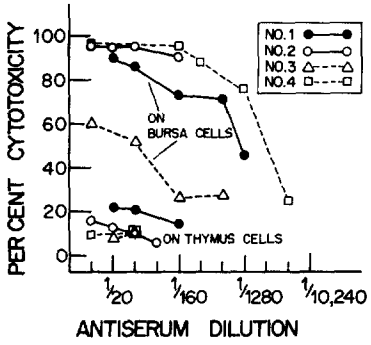

FIG. 2. Degree of cytotoxicity of various dilutions of different absorbed rabbit anti-bursa sera on chicken thymus and bursa cells.

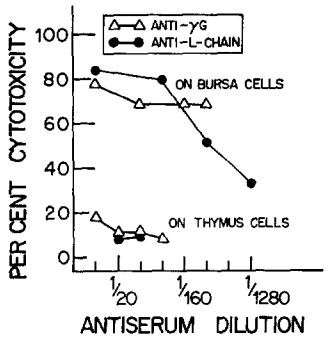

FIG. 3. Degree of cytotoxicity of various dilutions of different absorbed rab bit anti-immuno globulin sera on chicken thymus and bursa cells.

C′, or with medium alone, to below 30%; however, in an occasional experiment where a background of 10% was obtained, the percentages killed by the antisera tested were exactly similar to the ones listed in the table. The anti-thymus sera failed to significantly affect either DPFC or IPFC while killing a high percentage of spleen cells left unaffected by the anti-thymus sera (Table I).

Anti-immunoglobulin sera were highly cytotoxic for DPFC and somewhat less active against IPFC. Both antisera killed a low percentage of spleen cells above background, in the same range as anti-bursa sera.

DISCUSSION

These data clearly demonstrate that chicken thymocytes and bursa cells each possess at least one tissue-specific antigen on their cell surface. This was also suggested by the results of Forget et al. (24), although the percentage of cells in each organ carrying the specific antigen was not evaluated by these authors. The results of the cytotoxicity tests presented here strongly suggest that the antigen is present on >90% of thymus cells and that it is relatively easy to obtain a specific antiserum to this antigen in rabbits. The bursa-specific anti-

TABLE I

Cytotoxicity of Rabbit Antisera for Splenic Plaque-Forming Cells

Serum for incubation*	Spleen cells	Plaque-forming cells (PFC)‡	
		Direct	Indirect
	%	%	%
Normal	10–29	0	0
Anti-T1	52	10	12
Anti-T2	57	10	11
Anti-T3	64	14	18
Anti-T4	38	14	0
Anti-B1	28	33	72
Anti-B2	38	22	65
Anti-B3	35	14	43
Anti-B4	33	86	90
Anti-Ig	40	69	32
Anti-L	33	77	44

* The results represent the average percentages of killed cells obtained from several experiments after incubation with antisera diluted 1:20 and guinea pig C′ diluted 1:12.

‡ Tested 5–10 days after intravenous injection of SE. Ratio of indirect to direct PFC ranged from 10 to 25.

sera are somewhat more difficult to obtain, but the results with the two best anti-bursa sera suggest a representation of this antigen on >90% of bursa cells.

The thymus-specific antigen is similar to the murine θ (10), Ly (13), and mouse specific lymphocyte antigen (MSLA) (25), which are not only on all thymus cells but also on peripheral T-cells. The MSLA antigen is the most comparable since it is recognized by heterologous antisera rather than by alloantisera. It is, therefore, proposed to name the antigen chicken T-lymphocyte antigen (CTLA). It should be noted that the thymus-specific antigen seen on >90% of rat thymocytes (26, 27), although recognized by a heterologous antiserum, is different since it cannot be found on peripheral lymphoid cells.

The bursa-specific antigen is probably comparable to the one recently recognized by Raff (28) on murine B-cells, also by means of a rabbit antiserum, and called mouse B-lymphocyte antigen (MBLA). The similarity depends again on the presence of this antigen on peripheralized lymphoid cells as well as on the precursor cells in a central lymphoid organ. Although it was somewhat difficult to demonstrate a significant representation of cells with this antigen in the spleen, because of the high background mortality among spleen cells, there was a consistent increase in cytotoxicity over background with at least two of the anti-bursa sera as well as a significant effect on antibody-forming cells. In addition, the correlation with the effects obtained with antisera to chicken immunoglobulin suggested by others to be a surface marker for B-cells (14, 29, 30, 31)[2] was striking. It seems clear that the percentage for T-cells in chicken spleen is higher than that for B-cells, which is more like the murine lymph node than like the mouse spleen where B-cells are in the majority (14).[2] Since bursa lymphoid cells are derived from stem cells originating in hematopoietic tissue in the chick embryo (2), it may be safe to call the B-cell antigen detected by anti-bursa sera chicken B-lymphocyte antigen (CBLA) in conformance with the terminology used by Raff (28); however, at present we prefer to use the name chicken bursa-derived lymphocyte antigen (CBuLA) to caution against the temptation to equate bursa and bone marrow B-cells, particularly in view of the lack of evidence on cell-to-cell cooperation between bursa and thymus cells in the chicken (8). No effort has been made as yet to determine whether these antigens are present on other organs in the chicken, and representation of the antigen on peripheral lymphoid cells other than spleen has not been evaluated.

It is clear from other studies (18) that gross contamination of the chicken thymus with B-cells, in the form of germinal centers and plasma cell aggregates, increases with age. The chickens used in the present study were 4 wk of age or younger, and the upper limit of contamination with B-bells estimated for the thymus was only 0–6% as estimated with the two most potent anti-bursa sera. Conversely, the contamination of bursa with T-cells, as estimated with the most completely absorbed but still quite potent anti-thymus sera, was only 4% above background cytotoxicity levels. Low levels of cross-contamination between bursa and thymus were also indicated by the findings of Potworowski et al. (32).

The alloantigenic marker on murine antibody-forming cells, PC. 1 (33), was shown not to be present on precursor B-cells (9), whereas immunoglobulin surface markers are present on the majority, if not all, of murine B-cells (14)[2] and correspond in distribution to the MBLA marker (28). In the present study, cytotoxicity of anti-immunoglobulin sera for bursa cells was never as high (maximal 80%) as that of anti-bursa sera (>90%) suggesting that many but perhaps not all bursa cells have both the CBuLA and Ig marker.

It was somewhat surprising that the per cent inhibition for DPFC by anti-Ig was higher than for IPFC while the reverse was true for anti-bursa sera. It seems possible that some DPFC can be demonstrated even after the cells have been killed, simply by leakage of highly lytic antibody from recently killed cells. Such is not likely to occur when anti-Ig sera are used, which would not only kill the cells but would also bind the antibody within the dead cells.

Preliminary results suggest that the antisera to chicken thymus and bursa are extremely useful for the study of cellular aspects of the immune response in the chicken (34). Further studies on the biological effects of these antisera are in progress.

SUMMARY

Specific antisera to chicken thymus and to bursa of Fabricius were obtained in rabbits. After appropriate absorption and dilution all four anti-thymus sera, in the presence of guinea pig C′, killed >90% of thymus and ≦12% of bursa cells. They were cytotoxic for approximately 50% of spleen cells and did not affect antibody-forming cells. The surface antigen detected by these antisera was named chicken T-lymphocyte antigen (CTLA).

Two of four anti-bursa sera, under similar conditions, killed >90% of bursa cells and ≦10% of thymus cells. These antisera were cytotoxc for a large percentage of antibody-forming cells and killed approximately 30% of spleen cells The other two anti-bursa sera were somewhat less potent but showed similar specificity. The surface antigen detected by these antisera was named chicken bursa-derived lymphocyte antigen (CBuLA).

Rabbit antisera to chicken immunoglobulin were cytotoxic for bursa but not for thymus cells and killed a similar percentage of spleen cells as did anti-bursa sera. They were also cytotoxic for antibody-forming cells.

We are extremely grateful to Dr. A. A. Benedict for his generous gift of purified chicken L-chains. The valuable advice of Doctors M. W. Chase (The Rockefeller University, New York) and T. Takahashi (Sloan-Kettering Institute for Cancer Research, New York) is greatly appreciated.

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