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T and B Lymphocytes

ORGAN AND AGE DISTRIBUTION IN THE CHICKEN

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Summary. The occurrence of T and B lymphocytes was investigated by immunofluorescence in lymphoid organs of the chicken at different ages, using specific antisera. It was found that T lymphocytes make up most of the thymic cells in 2-week-old chickens and only about one half in chickens of 9 weeks and more. B lymphocytes account for approximately 1/3 of bursal cells in animals from 0 to 9 weeks. T and B lymphocytes in the spleen seem to lose some of their specific antigenicity.

INTRODUCTION

Cell-membrane antigens have been among the most successful markers used to identify functionally distinct lymphocyte populations.

In the mouse, thymus-derived lymphocytes carry the θ antigen (Raff, 1969; Schlesinger and Yron, 1969) while thymus-independent lymphocytes can be identified by a mouse-specific bone marrow-derived lymphocyte antigen (MBLA) (Raff, Nase and Mitchison, 1971). In the rat, three antigenically distinct lymphoid populations have been described: the first, characterized by a thymocyte-specific antigen was found only in the thymus (Potworowski and Nairn, 1967a), the second was composed of most of the peripheral lymphocytes and carried a lymphoid-specific antigen, while the third, found in the bone marrow, carried neither of these antigens (Potworowski and Nairn, 1968). In the chicken, lymphocytes from the bursa of Fabricius and from the thymus have been distinguished by antisera directed against a thymus-specific (T) and a bursa-specific (B) antigen respectively (Forget, Potworowski, Richer and Borduas, 1970). It was observed that the reactivity of the anti-B and anti-T sera was not limited to bursal and thymic cells but extended to peripheral bursa-dependent and thymus-dependent cells as well. Thus, only the anti-B serum could inhibit haemolytic plaque formation by sensitized spleen cells (Potworowski, Richer, Forget and Borduas, 1971) while only the anti-T serum could diminish the graft-versus-host reaction by buffy coat cells (Potworowski, Zavallone, Gilker and Lamoureux, 1971).

In the present work, the occurrence in different lymphoid organs of T and B lymphocytes was investigated in chickens of different ages in order to establish the cellular composition of these organs at different stages of their ontogeny.

MATERIALS AND METHODS

Animals

Outbred Leghorn chickens of both sexes aged 0, 2, 4, 6, 12 and 14 weeks and 14-day

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embryos (1 week before hatching) were used. Two animals made up each age group except for the −1 week group which was composed of two pools of three embryos each.

The tests were spread over a period of 3 months and were performed in random order so as to avoid a systematized error which could have been caused by a possible gradual loss of antibody activity of the antisera.

Cell preparations

The animals were killed by chloroform. Their thymus, bursa of Fabricius and in some cases their spleen were excised, thoroughly rinsed in Hanks's balanced salt solution, dissected free of fat and capsule and transferred into a washing medium containing 20 ml of 20 per cent bovine serum albumin, 50 ml phosphate buffered saline (0·01 M phosphate pH 7·1), 8 ml 5 per cent aqueous EDTA, 100 units penicillin/ml, $NaHCO_3$ to pH 6·8 (van Furth, Schuit and Hijmans, 1966). The organs were chopped with fine scissors and the milky suspension thus obtained was centrifuged at 500 ***g*** for 10 minutes. The supernatant, containing subcellular particles, and cell sap, was discarded and the sedimented cells were resuspended in approximately one ml of fresh washing medium. In the case of spleen cell suspensions, only the top layer of white cells ('buffy coat') was harvested after the centrifugation. The suspensions were filtered on a few strands of nylon wool placed in the gullet of a pasteur pipette, in order to rid them of large aggregates. The free cells were then transferred onto microscope slides using the technique and sedimentation apparatus described by Hijmans, Schuit and Klein (1969). The preparations were fixed in a mixture of 95 per cent absolute ethanol and 5 per cent glacial acetic acid at −30° for 15 minutes and kept in phosphate buffered saline at 4° until used.

Antisera

Techniques of immunization, serum absorption and specificity determinations have been described elsewhere in detail (Forget *et al.*, 1970). Briefly, two doses of 10^8 viable lymphoid cells in Hanks's balanced salt solution from chickens 1 to 3 week old were injected into a rabbit. The first dose was mixed with Freund's complete adjuvant and injected into the four footpads; the second dose was injected intravenously 2 weeks later. The rabbits were bled 7 days after the second injection and crude globulin fractions were obtained by ammonium sulphate precipitation. These antibody preparations were absorbed with chicken erythrocytes, serum and lymphocytes (bursal cells in the case of the anti-thymus serum and thymic cells in the case of the anti-bursa serum). The absence of antibodies to chicken serum proteins was verified by gel immunoelectrophoresis and the organ-specificity of the antibodies was determined by immune cytoadherence on young chicken thymus and bursal cells.

Immunofluorescence

The sandwich immunofluorescence technique was used with commercial goat-anti-rabbit globulin conjugated to fluorescein isothiocyanate (Hyland). The conjugate was thoroughly absorbed with chicken thymic and bursal cells until it gave no non-specific staining when used alone. In a typical test, three smears were made from both the thymus and the bursa of one animal: one was treated with anti-thymus serum, one with anti-bursa serum and one with normal rabbit globulin. The cell preparations were observed under a Reichert Zetopan fluorescence microscope fitted with a Tiyoda super-wide darkfield condenser and a 40× objective. Only cells appearing morphologically intact at

that magnification were counted. This was relatively easily achieved for the thymus but was more difficult for the bursa of Fabricius particularly in young animals. In the case of the bursa, rapid and gentle handling of the cells was crucial in obtaining clean preparation with minimal cell damage.

Negative cells could easily be identified by their low background fluorescence. For each preparation, the percentage of clearly fluorescent cells was determined.

Immunofluorescence on suspensions of living cells was attempted, but the extreme fragility of fowl lymphocytes compared to mammalian lymphocytes, caused such a high background of damaged cells that counting of fluorescent cells would have been meaningless.

RESULTS

The percentages of cells stained by the anti-T and the anti-B sera in thymus preparations from animals at different ages are reported in Fig. 1. In positive cells the pattern of staining

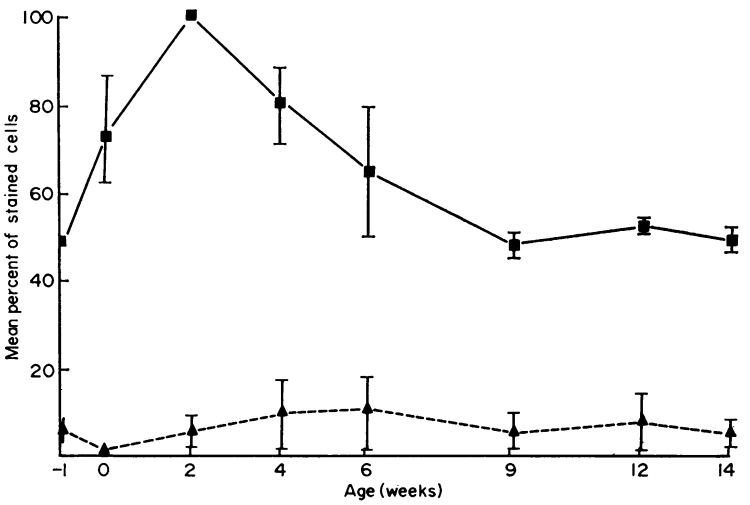

FIG. 1. Distribution of T cells (solid line) and B cells (dotted line) in the thymus of chickens at different ages.

was a continuous fluorescent ring. It was found that at 1 week before birth only about one half (46·5 per cent) of the thymic cells had the T antigen. This proportion gradually increased until it reached a peak at 2 weeks, when almost all (98 per cent) of the cells were T-positive.

The relative number of T-positive cells then decreased (78 per cent at 4 weeks, 68·5 per cent at 6 weeks) until the 9th week (47·5 per cent) at which level it seemed to remain fairly constant.

Thymic cells carrying the B antigen were rare throughout life, the highest level reached being 11·5 per cent at 6 weeks.

The general situation in the bursa of Fabricius was somewhat similar but less clear-cut (Fig. 2).

The level of bursal lymphocytes bearing the B antigen remained rather low throughout life: from 6 per cent in the 14-day embryo, it reached 35 per cent at two weeks and stayed approximately at that level (30 per cent at 4 weeks, 31·5 per cent at 6 weeks), until week 9 (42 per cent), at which time it dropped again.

In the bursa of Fabricius, the proportion of T lymphocytes remained low: from 0 per cent in the embryo it never went above 15 per cent. This maximum was attained in the 6-week-old chicken.

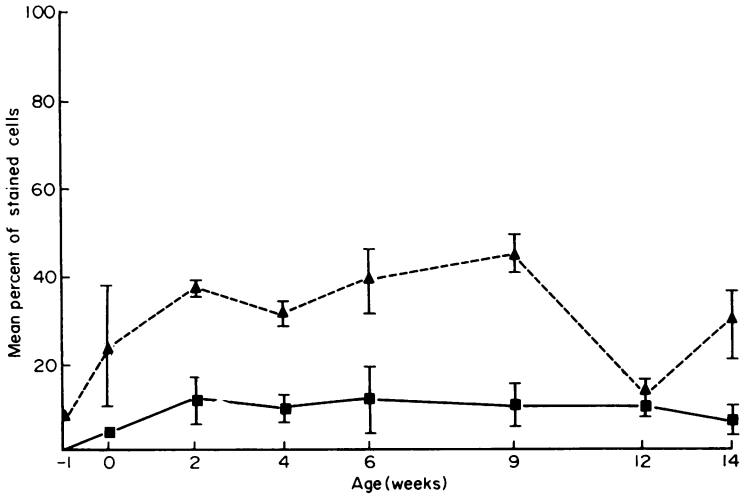

FIG. 2. Distribution of T cells (solid line) and B cells (dotted line) in the bursa of Fabricius of chickens at different ages.

It is important to note that up to about 9 weeks, both in the thymus and in the bursa, the cells could easily be classified into either strongly fluorescent or clearly non fluorescent. In older animals, this distinction could no longer be made with such ease as those cells which were fluorescent gave a much weaker staining. For that reason, cell counts were not made in animals older than 14 weeks, as the error would have been too great.

Similarly, some spleen cells of animals at all ages gave weak staining with both the anti-T and the anti-B sera, so that no precise counts could be made in that organ. It was possible to note, however, that in all age groups, approximately 1/3 of the cells stained with the anti-T serum and the same proportion were positive with the anti-B serum. Both antisera reacted with some plasma-like cells, but never with all. When both anti-T and anti-B sera were used simultaneously on the same spleen preparation, it was estimated that approximately two thirds of the cells were stained, indicating that the two staining populations were, at least to a large extent, mutually exclusive. Owing to the weak staining, our observations were not sufficiently precise to exclude the possibility of some degree of overlap. Even in the doubly-stained spleen preparations, one third of the lymphocytes and some plasma-like cells were still negative. In similar spleen preparations treated with fluorescein-conjugated goat anti-chicken globulin some cells were weakly fluorescent but the staining pattern was patchy and resembled that reported by Kincade, Lawton and Cooper (1971). In these preparations, some of the plasma-like cells referred to earlier were negative.

Spleens of animals in a state of delayed hypersensitivity to BCG did not give a different proportion of positive cells with either of the antisera, nor did neonatally bursectomized animals examined at four weeks. However, in the spleens of neonatally bursectomized animals, sensitized to BCG later in life, two thirds of the cells were weakly fluorescent with the anti-thymus serum and one third with the anti-bursa serum.

In the bone-marrow, the majority of cells morphologically identified as small lymphocytes were negative to both sera: less than 10 per cent gave detectable fluorescence with the anti-bursa serum and even fewer (0·5 per cent) with the anti-thymus serum. Most of the large mononuclear cells with abundant cytoplasm were weakly fluorescent with the anti-thymus and some were also positive to the anti-bursa serum. Although no precise count could be made because of the faintness of the staining, an overlap here is possible.

DISCUSSION

Our results indicate that there are at least three antigenically distinguishable lymphoid populations in the chicken; the first is found in greatest concentration in the 2-week-old thymus (T cells), the second is found primarily in the bursa of Fabricius (B cells). B and T cells are also found in the spleen in varying proportions but in cells of that organ the concentration of antigens is less than in either the thymus or the bursa. Cells carrying neither T nor B antigen could be identified in spleen preparations stained simultaneously with both antisera. Such non-reactive cells were also found in the bone marrow, in the bursa of Fabricius and in the thymus of embryos and of animals older than 2 or 3 weeks. The 'contamination' of the bursa with T cells and of the thymus with B cells only accounts for a small proportion of the non-native lymphocytes. Whether those non-reactive cells form one homologous population is not known; they could well resemble the 'null' lymphocytes described by Stobo (personal communication) in the mouse, which carry neither the θ antigen nor immunoglobulin determinants.

The gradual loss of thymic antigen from the thymus after birth has already been described in the rat (Potworowski and Nairn, 1967b) and in the mouse (Stutman, 1971, unpublished observations), and probably represents a normal ageing process of the immunological system.

Several possible mechanisms can be postulated to explain the diminishing proportion of T cells from the thymus: it could well be due to the increasing size of a non-T population; the absolute number of T cells could then remain the same or even increase at a slower rate than the non-T population and their proportion would still be decreasing. Independently of this possibility, the now well-documented (Parrott and de Sousa, 1971) emigration of T lymphocytes from the thymus must also be considered. The observation, however, that the decreasing proportion of T cells from the thymus did not correspond to an increase of these cells elsewhere must be due to a partial loss of antigenicity concurrent with their emigration from the thymus. Such a change in antigenicity from thymocyte phenotype to that of a peripheral thymus-derived cell has already been reported in the mouse, where a partial loss of TL antigen occurs in peripheral organs (Lance, Cooper, Buchhagen and Boyse, 1970; Raff, 1971). If such is the case with chicken T and B cells, the weak fluorescence of cells in the spleen and in the thymus and bursa of older animals could be interpreted as a partial loss of T and B antigens.

Our results could provide an explanation for the low immune adherence titres obtained with anti-B serum reacting with bursal cells (Forget *et al.*, 1970) of which only 1/3 could

be identified as B cells by immunofluorescence. The observations reported here also emphasize the importance of choosing lymphoid organs from animals of the appropriate age for preparing specific antisera as well as for absorbing these antisera (Jankovic, Isakovic, Petrovic and Vujic, 1970; Wick, Kite and Cole, 1971). The diversity of the ages of lymphoid organs used for preparing ALS and the resulting differences between batches both in cytotoxic titres and *in vivo* reactivity could well be due to different proportions of antigenically distinct lymphoid populations injected each time.

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REFERENCES

Forget, A., Potworowski, E. F., Richer, G. and Borduas, A. G. (1970). 'Antigenic specificities of bursal and thymic lymphocytes in the chicken.' *Immunology*, **19**, 465.

Hijmans, W., Schuit, H. R. E. and Klein, F. (1969). 'An immunofluorescence procedure for the detection of intracellular immunoglobulins.' *Clin. exp. Immunol.*, **4**, 457.

Jankovic, B. D., Isakovic, K., Petrovic, S. and Vujic, D. (1970). 'Studies on antilymphocyte antibody in the chicken. I. Effect of rabbit anti-thymus and antibursa globulin on chicken embryos.' *Clin. exp. Immunol.*, **7**, 693.

Kincade, P. W., Lawton, A. R. and Cooper, M. D. (1971). 'Restriction of surface immunoglobulin determinants to lymphocytes of the plasma cell line.' *J. Immunol.*, **106**, 1421.

Lance, E. M., Cooper, S., Buchhagen, D. and Boyse, E. A. (1970). 'Thymus leukemia (T1) antigen and thymocyte maturation.' *Fed. Proc.*, **29**, 436.

Parrott, D. M. V. and de Sousa, M. (1971). 'Thymus-dependent and thymus-independent populations: origin, migratory patterns and lifespan.' *Clin. exp. Immunol.*, **8**, 663.

Potworowski, E. F. and Nairn, R. C. (1967a). 'Specific antigenicity of thymocytes.' *Nature* (*Lond.*), **213**, 1136.

Potworowski, E. F. and Nairn, R. C. (1967b). 'Origin and fate of a thymocyte-specific antigen.' *Immunology*, **13**, 597.

Potworowski, E. F. and Nairn, R. C. (1968). 'Lymphoid-specific antigen: distribution and behaviour.' *Immunology*, **14**, 591.

Potworowski, E. F., Richer, G., Borduas, A. and Forget, A. (1971). 'Selective inhibition of plaque-forming cells by bursa-specific antisera.' *J. Immunol.*, **106**, 1416.

Potworowski, E. F., Zavallone, J. D., Gilker, J. C. and Lamoureux, G. (1971). 'Inhibition of the graft-versus-host reaction by thymus-specific antibodies.' *Europ. J. clin. biol. Res.*, **16**, 155.

Raff, M. C. (1969). 'Theta isoantigen as a marker of thymus-derived lymphocytes in mice.' *Nature* (*Lond.*), **224**, 378.

Raff, M. C. (1971). 'Evidence for subpopulation of mature lymphocytes within mouse thymus.' *Nature New Biology*, **229**, 182.

Raff, M. C., Nase, S. and Mitchison, N. A. (1971). 'Mouse specific bone marrow derived lymphocyte antigen as a marker for thymus-independent lymphocytes.' *Nature* (*Lond.*), **230**, 50.

Schlesinger, M. and Yron, I. (1969). 'Antigenic changes in lymph node cells after administration of antiserum to thymus cells.' *Science*, **164**, 1412.

Van Furth, R., Schuit, H. R. E. and Hijmans, W. (1966). 'The formation of immunoglobulins by human tissues *in vitro*. I. The methods and their specificity.' *Immunology*, **11**, 1.

Wick, G., Kite, J. H. and Cole, R. C. (1971). 'Suppression of spontaneous autoimmune thyroiditis in obese strain (OS) chickens with anti-bursa and anti-thymus sera.' *Int. Arch. Allergy*, **40**, 603.