# ANTIGEN-INDUCED CHANGES IN LYMPHOID CELL HISTONES

## I. Thymus

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ABSTRACT

An acute effect of antigens on the nuclear histones of mouse thymocytes was investigated by means of cytophotometric measurements of thymocytes stained with ammoniacal-silver (A-S) and with fast green (FG). In addition, the DNA content was measured in terms of Feulgen staining. In terms of such staining it appeared that nuclei of control thymocytes contain a greater amount of nuclear histones and a higher histone/DNA ratio than do renal cell nuclei from the same animal. Within 1 hour after the injection of antigen the thymocyte nuclei appear to lose approximately 32 per cent and 20 per cent, respectively, of A-S and FG stainable nuclear proteins, while the Feulgen staining remains unchanged. Since the renal cell nuclei show no antigen-induced change in histone staining, the histone staining and histone/DNA ratios were found to be similar in the thymocytes and renal cells of the antigen-injected mice. The antigen-induced loss of thymocyte histones was also found to be associated with a change in the color of the A-S staining, from yellowish brown to black. This and other findings suggest that thymocyte nuclei contain an antigen-labile, lysine-rich histone. The implication of these observations in regard to the phenomenon of immunological competence is discussed and the need for continued investigation indicated.

## INTRODUCTION

In previous studies we had observed antigen-induced changes in the ammoniacal-silver (A-S) staining of nuclear chromatin of lymphoid cells (1, 2). Subsequent studies demonstrated that under controlled conditions A-S staining visualizes nuclear histones (3, 4). In a further investigation of the influence of antigens on the nuclear histones of lymphoid cells we have utilized the more conventional alkaline fast green (FG) method for the detection and measurement of histone, in addition to the A-S reaction. This report presents observations on the microspectrophotometric analysis of the nucleoprotein of mature thymocytes, before and after the administration of antigen. Subsequent papers will describe the findings in other lymphoid structures.

## MATERIAL AND METHODS

The present study employed male and female CF-1 mice (Carworth Farms, New City, New York) weighing 15 to 20 gm. In the majority of instances in this study the test animals were injected intraperitoneally with 0.1 ml of tetanus toxoid (Wyeth) and sacrificed at various times after the injection of antigen. Thus, groups of 3 to 5 animals were sacrificed at 30 minutes and at 1, 2, 3, 4, 5 and 24 hours after an intraperitoneal injection of the tetanus toxoid, and measurements were made on each animal. Such observations were also made on groups of

animals sacrificed 1 hour after the intraperitoneal injection of tetanus toxoid diluted 1 to 10 and also after the injection of foreign serum (human and goat) and tuberculin. Additional measurements were made after intravenous and subcutaneous injections of tetanus toxoid.

Unless specified otherwise, the results presented below are those obtained after intraperitoneal and intravenous injection of the antigen, since these two routes of administration yielded similar results. On the other hand, the effects of subcutaneous injection were found to vary with the injection site and the concentration of the injected antigen. The detailed observations on the effects of subcutaneous injections will be the subject of a subsequent report.

After sacrifice, the thymus, spleen, kidney, liver, lymph nodes, and other organs were rapidly removed for study. The tissues were smeared on standard microscope slides and dried at room temperature. Alternate smears of a given organ were stained with A-S, FG, and the Feulgen procedure. Tissues to be compared cytophotometrically, *e.g.* tissues from iniected and uninjected animals, were stained together in the same Coplin jar.

The fast green staining was done according to the directions of Alfert and Geschwind (5) and Godman and Deitch (6).

For A-S staining the procedure was as follows: All slides were placed in 10 per cent formalin for 1 hour at 26–28°C. The formalin solution was acetate-neutralized formalin routinely used for surgical specimens in the Department of Pathology. It was customarily prepared in 10-liter amounts by adding 200 gm of sodium acetate to 1 liter of formaldehyde and diluting to 10 liters with tap water. The pH of the solution was 7.1. After treatment with formalin, the slides were washed vigorously in five changes of distilled water and immersed with agitation in 10 per cent ammoniacal-silver for 10 seconds. The A-S solution was prepared by the dropwise addition of 10 per cent silver nitrate to 4 ml of concentrated ammonium hydroxide until a persistent turbidity was obtained. The slides were again washed in five changes of distilled water and developed for 2 minutes in 3 per cent formalin (prepared from the fixative). The slides were then washed, dehydrated, and mounted in Permount. It should be noted that the prestaining exposure to formalin is crucial to the success of the stain. The result varies widely with temperature, pH, and composition of the formalin bath. For instance, tap water provides a mixture, apparently, of trace elements or ions which permits the formalin pretreatment to be as short as 1 hour, but in the absence of this unknown quantity in New York City tap water (*i.e.* after using distilled water instead of tap water), the pretreatment must be prolonged for a period of at least 12 hours.

The Feulgen procedure followed that of Bloch and Godman (7).

The relative intensity of staining before and after the injection of antigen was measured microspectrophotometrically according to the method of Pollister (8, 9). The apparatus employed was manufactured by Canalco (Bethesda, Maryland) and consisted primarily of a digital ratio computer receiving simultaneous impulses from two 1-P-21 photocells, one of which sampled light as it emerged from a grating monochromator and the other of which measured the same light after it had passed through the object or specimen as visualized by the microscope. The slits of the monochromator were set to resolve 20 A of the visible spectrum. Especially well matched photocells having low dark current and high red sensitivity were utilized. Various tests devised by Pollister and Ornstein (10) were applied to determine that the system was well aligned and functioning without flare. Repeated measurements of the same cell revealed a constant reproducibility to within better than 0.2 per cent of the transmission reading.

The only lymphoid cell type for which data are presented here is the mature thymocyte. In our material this class of cell showed almost negligible variation in size, the average nuclear diameter being 5 $\mu$. The kidney provided an excellent control material since certain tubular cells presented the same nuclear diameter.

The two-wavelength method of Ornstein (11) was employed for Feulgen and fast green preparations, using Mendelsohn's tables (12). Feulgen-stained material was measured at 502 and 550 m$\mu$; fast green was measured at 572 and 600 m$\mu$. The A-S–stained material was measured at 480 m$\mu$ by the plug method. This wavelength was found to approximate maximal absorption for all types of cells stained by A-S (Fig. 1). The plug size was kept as large as possible, up to 90 per cent of the area of the nuclei being included.

## RESULTS

### *Thymus*

The nuclei of the thymocytes were stained alternatively by FG, A-S, and the Feulgen procedure. In optical section, mature thymocytes are characterized by a centrally located chromatin condensation or false nucleolus which stains prominently by all three methods (Figs. 2 and 3). A-S–stained preparations may also be subsequently stained by the Feulgen procedure. Such doubly stained preparations reveal that the A-S and Feulgen procedures stain the same chromatin structures. Microspectrophotometric measurements of Feulgen-stained thymocyte nuclei yielded a mean relative value of 0.550 $\pm$ 0.002 and 0.549

± 0.002 in control and antigen-injected groups, respectively.

AMMONIA CAL-SILVER STAIN: The A-S staining was found to be altered qualitatively as well as quantitatively after antigen injection. The nucleoprotein of normal thymocytes, for example, presents a yellowish brown color when stained with A-S. This yellow-brown coloration is typical of normal lymphoid cells in general, in contrast to cells of liver, kidney, brain, and connective tissue, the nuclei of which normally stain black with A-S under the same conditions. Within an hour after the injection of an antigen the color of thymocyte nuclei becomes grayish or black, corresponding to the rest of the body tissues. The change in the absorption characteristics of the A-S stain as a result of antigen injection is shown in Fig. 1.

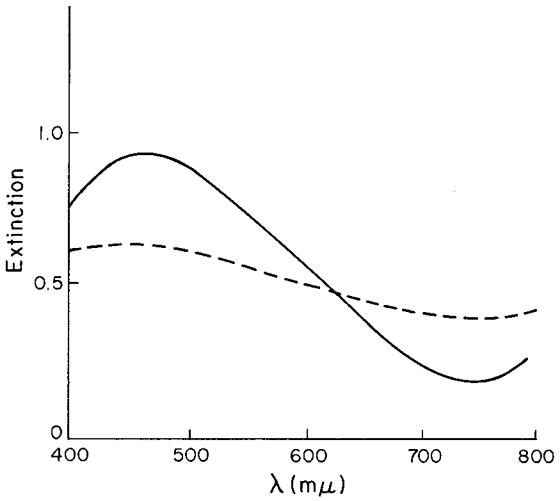


FIGURE 1 Absorption curves of representative A-S–stained thymocytes from control (solid line) and antigen-injected mice (broken line). The nucleus of control thymocytes has a yellowish brown color, the extinction of which varies with the wavelength, with a maximal absorption between 460 and 500 mμ. Nuclei of thymocytes from antigen-treated animals typically have a black color similar to that of kidney and other non-lymphoid cell nuclei. Extinction values of such cells show less variation with wavelength.

Although the A-S reaction has the great advantage of indicating qualitatively the lymphoid cell response to antigen, it also lends itself to quantitative evaluation.

The frequency distribution of microspectrophotometric measurements of A-S–stained control thymocytes is given in Fig. 4. The mean relative value (extinction × area) was 0.601 ± 0.003 (SE). After antigen injection a marked decrease of A-S nuclear staining was noted. A similar effect was found to be produced by tetanus toxoid, full strength or diluted 1:10, administered intraperitoneally or intravenously. Such results were also obtained when foreign serum protein and tuberculin were employed. Such changes are initiated within 30 minutes, are fully developed within 1 hour after the antigen injection, and are maintained through the 5-hour period. The mean relative value of the A-S–stained thymocytes during the 1- through 5-hour period was 0.410 ± 0.004 (SE).

As depicted in Fig. 2 *b*, these changes include an apparent loss of much of the diffuse staining of the nucleoplasm as well as a decreased intensity of staining of the chromatin clumps, which also assumes a more granular appearance. These changes are in addition to the change in the color, which becomes blackish rather than yellowish brown. However, 24 hours after the injection of antigen the thymocytes once again appear similar to control thymocytes in both color and amount of A-S staining.

FAST GREEN STAIN: An antigen-induced decrease in staining was also found in FG-stained preparations. As depicted in Fig. 3, the FG-stained chromatin of the antigen-affected thymocytes appears somewhat less distinct than that of control thymocytes. Such changes are detectable within 1 hour after antigen injection and persist through the 5-hour interval. During this period the mean relative amount of FG-stainable nuclear protein was 0.362 ± 0.002 in contrast to the control thymocyte value of 0.455 ± 0.003. As indicated in Fig. 4, the control and antigen-affected thymocytes were found to comprise two distinct populations in terms of their histone content. However, as was found in the A-S studies, the FG

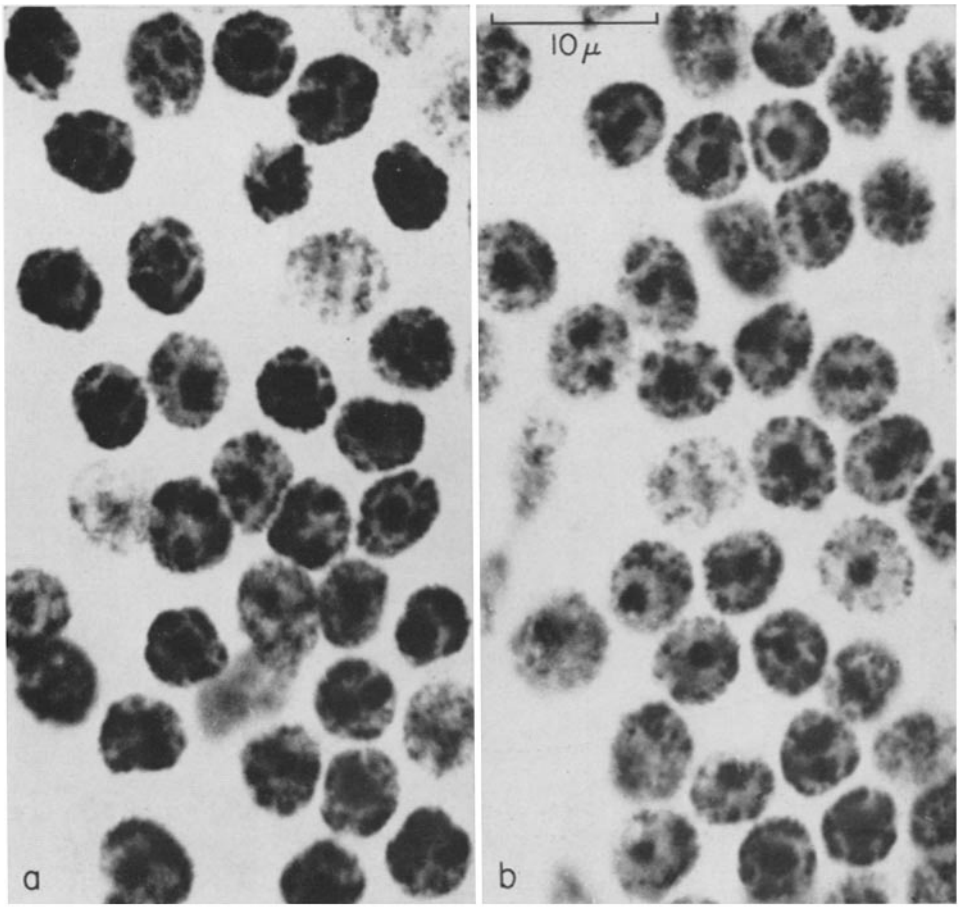


FIGURE 2 Smears of A-S–stained thymocytes from (*a*) control and (*b*) antigen-injected mice (tetanus toxoid) photographed under identical conditions using Kodak Plus X Pan film and tungsten lamp with blue filter. Nuclei of control thymocytes (*a*) are characterized by dense yellowish brown staining of chromatin clumps and nucleoplasm. Nuclei of antigen-affected thymocytes (*b*) stain blackish and show diminished, somewhat granular stain. Such diminished staining occurs in almost all of the mature thymocytes. Such changes are initiated within 30 minutes after the injection of antigen.

staining had returned to normal values 24 hours after antigen injection.

As shown in Fig. 5, the subcutaneous injection of tetanus toxoid is also associated with a decrease in FG and A-S stainable nuclear histone of thymocytes. In this study mice were injected subcutaneously with 0.1 ml of tetanus toxoid in the region of the upper forelimb. One group received undiluted antigen, while the other group received a 1:10 dilution of the antigen. All the mice were sacrificed 1 hour after the injection. It is evident that the diluted antigen caused a real but less marked loss of histone staining. It should also be noted that the effect of the subcutaneous injection of the full strength tetanus toxoid was found to resemble closely the effect produced by intraperitoneal and intravenous injections.

### *Kidney*

With A-S staining the nuclear chromatin of the renal cells of both the control and antigen-injected mice is typically stained black rather than yellow-brown. Both control and antigen-injected groups were found to contain the same relative amounts of histone as determined by both FG and A-S staining.

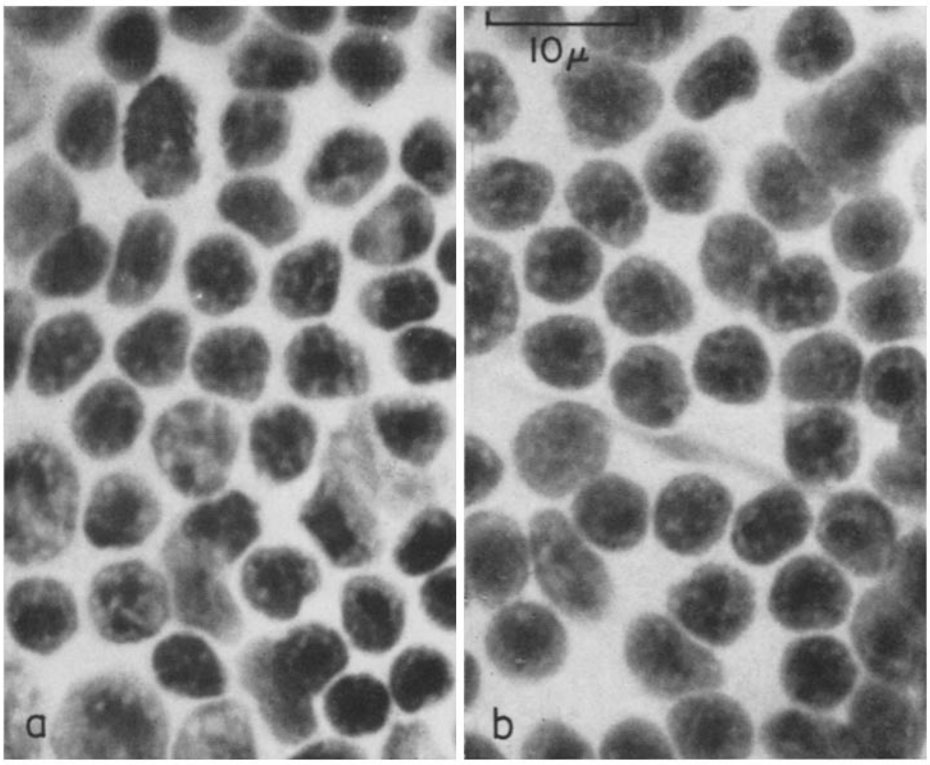


FIGURE 3 Mouse thymocytes stained with FG: (*a*) control; (*b*) injected intraperitoneally with tetanus toxoid 2 hours previously. Cytophotometric measurements disclose a decrease of 20 per cent in FG-stainable histone in antigen-affected thymocytes. Such changes are not so striking upon direct microscopic examination (as here) as the pronounced changes in the color and intensity of A-S–stained preparations.

Of particular interest was the finding that renal cell nuclei contain less A-S and FG stainable nucleoprotein than do nuclei of control thymocytes from the same mouse. The mean relative value for kidney tubule cell histone was found not to differ significantly from that for the antigen-affected thymocytes for both A-S– and FG-stained material (Fig. 4).

The DNA content (Feulgen staining) of the renal cell nuclei was found to be essentially the same as that of the thymocyte nuclei and was unaffected in either cell type by antigen injection. As indicated in Table I, the control thymocyte is characterized by a higher histone/DNA ratio than the renal tubular cell. After antigen injection, however, the histone/DNA ratio of the thymocyte nuclei becomes similar to that of the control renal cell nuclei.

## COMMENTS

The present study has disclosed a hitherto unrecognized response of lymphoid cells to antigens, namely an apparent discharge of histones. No such change occurs after the injection of saline. Nor have we observed any such changes in histone within 5 hours after the intraperitoneal injection of 5 milligrams of cortisone. However, it remains to be determined whether the apparent histone discharge from thymocytes is invoked only by antigenic stimuli.

The fact that two different methods of histone staining have both demonstrated decreased staining suggests that there is a real decrease in histone content. However, the possibility that the staining alterations might be due to the appearance of a masking non-histone protein or to a change in the reactive groups of the histone cannot be excluded. Nor do the present observations allow us to decide

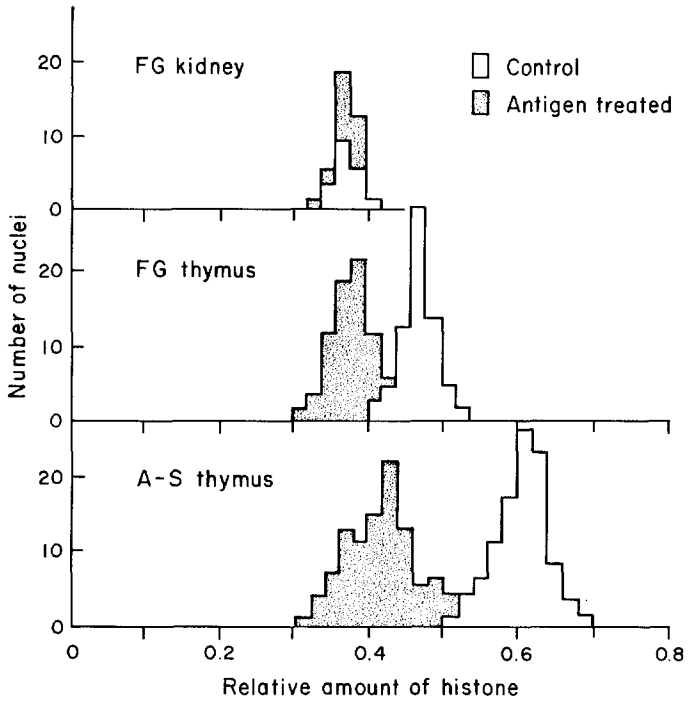


FIGURE 4 Frequency distribution of relative amount of ammoniacal-silver (A-S) and fast green (FG) stainable histone in thymocytes and renal tubular cells. An antigen-induced decrease in thymic histone is revealed by both A-S and FG staining procedures. Such changes contrast with the lack of alteration in the nucleohistone of renal cells. These data are representative of the findings during the period 1 to 5 hours after antigen injection.

whether the apparent decrease is due to discharge or degradation of the histones. It is nevertheless true that a change in the nucleohistones of thymocytes is the earliest cytochemical change in response to antigen recognized thus far, and that a sizable amount of thymocyte histone appears to be antigen-labile. It should also be mentioned that preliminary studies utilizing $C^{14}$ and tritium labeling of the thymocyte histones suggest that the antigen-induced decrease in histone staining is associated with a loss of histone from the nucleus. Furthermore, we have observed that the staining of the nuclei of antigen-affected thymocytes is restored to normal by incubating such cells in solutions of calf thymus histone, whereas the staining of control thymocytes is not affected by such treatment.

The fact that A-S staining was reduced more than FG staining by the antigen fits some of our other observations with respect to the two staining procedures. For example, A-S stains lysine-rich histones more readily than arginine-rich histones, whereas FG staining is less selective in this regard. Accordingly, the more pronounced change in A-S staining as compared with FG staining suggests a preferential loss of lysine-rich histones. This possibility is supported by the loss of the yellow-brown A-S staining of control thymocytes after antigen injection, since electropherograms of

TABLE I

*Histone/DNA Ratios in Thymocyte and Renal Tubular Cell Nuclei from Control and Antigen-Injected Mice**

| | Thymus | | Kidney | |
|---|---|---|---|---|
| | Contol | Antigen-injected | Control | Antigen-injected |
| A-S/Feulgen | 1.09 | 0.75 | 0.75 | 0.75 |
| FG/Feulgen | 0.84 | 0.66 | 0.66 | 0.66 |

* Histone measurements in terms of ammoniacal silver (A-S) and fast green (FG) staining. DNA measurements in terms of Feulgen staining.

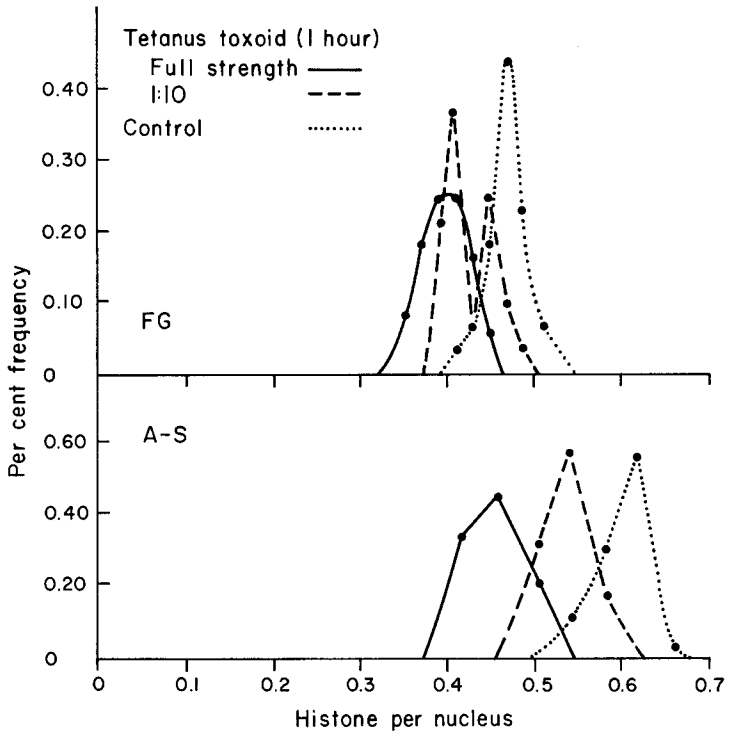


FIGURE 5 Per cent frequency distribution of relative amount of FG and A-S staining in thymocytes before and 1 hour after subcutaneous injection (upper forelimb) of tetanus toxoid, full strength and diluted 1:10. Note that the decrease in staining after subcutaneous injection of full strength tetanus toxoid was similar to that after intraperitoneal injection. An intermediate decrease in histone staining was produced by the diluted antigen.

thymus histones are characterized by a rapidly moving lysine-rich component which stains yellow-brown with A-S (13).

As will be reported subsequently, a similar antigen-induced decrease in staining of histones also occurs in the nuclei of lymphocytes of the spleen and lymph nodes. In these organs, however, the decreased staining of nuclear histones of the lymphoid cells is followed by an increased staining of the histones of reticular cells.

The data obtained in the present study raise provocative questions regarding the role of histones in the phenomenon of immunological competence. The release of histones from the nuclei of lymphoid cells might be a key feature in immunological responsiveness, since histones are highly reactive molecules which combine readily with proteins, polysaccharides, bacteria, and viruses. Furthermore, the increasing literature on the ability of histones to influence DNA-mediated messenger RNA synthesis suggests that a change in the histones of lymphoid cell nuclei might play a role in the subsequent differentiation of antibody-producing cells (14, 15).

It is also significant that tetanus toxoid and human serum produce similar effects on thymocyte nuclear histone and that both antigens appear to cause a loss of histones from all the thymocytes. This finding was confirmed by examining cryostat sections of thymus glands from control and antigen-injected mice. Goat serum and tuberculin also gave identical results when used as the antigen. Such preparations clearly demonstrate that the apparent loss of histones occurs in thymocytes throughout the cortex rather than in particular groups of thymocytes, as might be expected from some interpretations of the clonal hypothesis of antibody formation (16).

The methods used in the present study appear capable of revealing important cytochemical features involved in the earliest responses of lymphoid cells to antigens. Further studies are needed to define the significance of the observed changes for other aspects of immunological reactions, *e.g.* immunological paralysis, antibody formation, the role of the thymus in the development of immunological competence.

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