

A CELLULAR DEFICIT IN THE RECONSTITUTIVE CAPACITY OF  
IMMUNE POPULATIONS OF LYMPHOID CELLS  
DEMONSTRABLE IN STUDIES OF DELAYED  
HYPERSENSITIVITY IN MICE

EVIDENCE FOR THYMUS-BONE MARROW CELL SYNERGISM\*

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(Received for publication 17 December 1970)

Previous work has provided evidence that antigenic competition is associated with a relative deficiency in a thymus cell population created by a disproportionately marked proliferation of nonthymus cellular elements in the maximally stimulated lymphoid tissue (1).<sup>1</sup> Such cellular events were considered to alter the lymphoid micro-environment such that the chance occurrence of cellular interaction for an immune response to the second test antigen in models of antigenic competition was deficient. The descriptive term non-specific antigen-induced suppression (AIS)<sup>2</sup> was coined to replace the term antigenic competition.

In the present work, experiments comparing the efficacy of immune and normal<sup>3</sup> populations to reconstitute the immunological reactivity of lethally irradiated mice were carried out employing various populations of cells, and utilizing methylated human serum albumin (MHSA) as the test antigen for the development of delayed hypersensitivity. The reasons for the choice of MHSA to test the development of delayed hypersensitivity may be enumerated as follows: (a) Methylated HSA has been shown to preferentially produce delayed hypersensitivity in mice (2). (b) The reaction develops as a consequence of intradermal, footpad injection of the antigen, and is characterized by induration and edema which can be easily and accurately measured employing a fine micrometer. (c) The likelihood of cross-reaction between the artificially altered serum protein and other antigens employed in models of AIS is diminished. (d) Preparation methods show the carboxyl groups of the native molecule to be virtually completely esterified (3), thus diminishing the possibility of the kind of heterogeneity of the immune response expected against the native molecule.

\* This work was supported by grants from the National Cancer Institute, the Ontario Heart Foundation, and the Medical Research Council of Canada.

<sup>1</sup> Kerbel, R. and D. Eidinger. Antigenic competition: a new hypothesis based on cell interactions in the immune response. Submitted for publication.

<sup>2</sup> *Abbreviations used in this paper:* AIS, antigen-induced suppression; MHSA, methylated human serum albumin.

<sup>3</sup> The designation "normal" population of cells refers to suspensions derived from animals not purposefully immunized with antigen. They would, of course, be derived from spleens variously stimulated by environmental antigens.

During the course of the experiments, it became clear that not only could the differences between immune and normal populations of cells be demonstrated, but also, that cell-transfer systems provided a unique opportunity to study the synergism between bone marrow and thymus cell populations in reconstitution of delayed responsiveness. Although some studies have been carried out pertaining to the role of thymus and bone marrow populations in reconstituting reactions of delayed hypersensitivity (4, 5), relatively little is known.

In the present work, it was shown that thymus and bone marrow cells act in synergism to reconstitute delayed responsiveness to MHSA. The reconstitutive capacity of an immune spleen cell population for lethally irradiated mice is deficient as a consequence of a deficit in numbers of the thymus cells. On the other hand, a bone marrow cell is the limiting cell in determining the degree of responsiveness induced by a fixed number of normal spleen cells.

#### *Materials and Methods*

*Mice.*—Female mice of the CAF<sub>1</sub>, and B<sub>6</sub>D<sub>2</sub>F<sub>1</sub><sup>4</sup> hybrid strains were used. Mice were 8–12 wk of age at the onset of experiments.

*Antigens.*—Methylated human serum albumin was prepared by the method of Crowle et al. (2). Appropriate suspensions of goose erythrocytes suspended in Alsever's were prepared as described elsewhere.<sup>5</sup>

*Irradiation.*—An Atomic Energy of Canada Limited gamma cell 20 irradiator was employed; the instrument was specifically designed for irradiation of small animals. The irradiation source was cesium 137 delivered at a rate of 66 rads/min whole body irradiation. The mice which were housed in a lucite chamber were generally irradiated with 850 R unless stated otherwise.

*Preparation of Cells.*—Cell suspensions derived from spleen, thymus, and bone marrow were prepared as previously described.<sup>5</sup> Donor animals were either normal adult mice or mice which had been immunized 3 days previously with goose erythrocytes. The choice was based on previous experiments indicating that goose erythrocytes yielded maximum immunosuppression of the response to an unrelated antigen in a model of AIS when the doses of the two antigens were administered sequentially at a 3 day interval (6).

Lethally irradiated recipients were given varying numbers of cells as will be described, after which they were sensitized to MHSA. For this purpose, 0.2 mg of MHSA emulsified with complete Freund's adjuvant contained in a volume of 0.05 ml of distilled water was injected into a single hind footpad.

9 days after reconstitution, recipients were challenged with 0.02 ml of a 1% MHSA in distilled water into the footpad contralateral to the side used for immunization. The diameter of the footpads was measured before and at different times after challenge as described by Halliday and Webb (7).

<sup>4</sup> Obtained from the Jackson Laboratories, Bar Harbor, Maine.

<sup>5</sup> Eiding, D., M. G. Baines, H. F. Pross, R. S. Kerbel, A. Ackerman, and S. A. Khan. 1971. Further studies on competition of antigens. I. Variation in immunosuppression induced by alterations of dosage, route of injection, nature of antigen, and immunological status of host. *Can. J. Microbiol.* In press.

## RESULTS

*Relationship between Number of Transferred Spleen Cells and Response to MHSA in Reconstituted CAF<sub>1</sub> Hybrid Mice.*—A comparative study of the capacity of normal spleen cells and spleen cells derived from donor mice immunized 3 days previously with goose erythrocytes to reconstitute lethally irradiated syngeneic recipients was carried out. Fig. 1 summarizes the data for both CAF<sub>1</sub> and B<sub>6</sub>D<sub>2</sub>F<sub>1</sub> hybrid mice. It may be seen that a marked deficiency in the reconstitutive capacity of immune cells was demonstrated at each cell

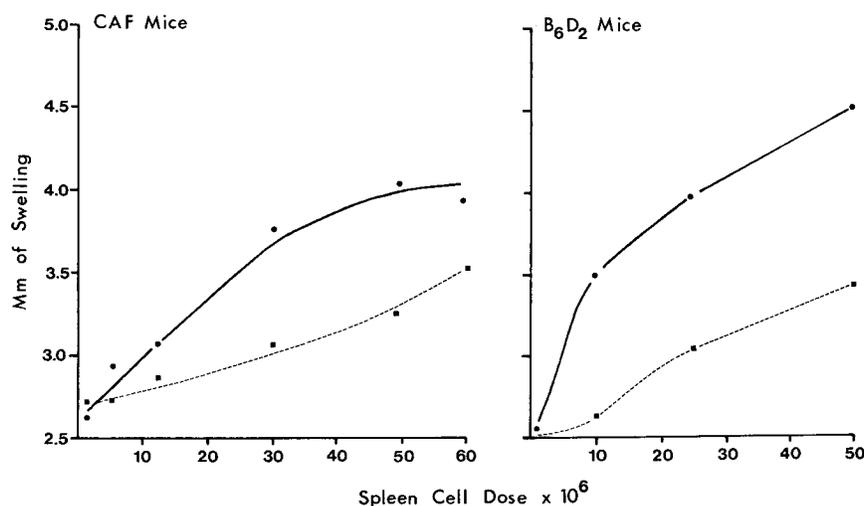


FIG. 1. Footpad swelling of groups of lethally irradiated recipient mice reconstituted with varying numbers of syngeneic normal (solid line) and immune (dotted line) spleen cells; the latter were obtained from donors immunized 3 days previously with 0.2 cc of 10% goose erythrocytes. Each point represents the mean diameter of swelling of groups of 10–21 mice of the two hybrid strains indicated.

concentration employed in the study. These differences did seem to level off at the higher cell doses due probably to the limitation in the swelling capacity of footpads at these cell doses. Some strain differences were also noted.

*The Reconstitutive Capacity of Various Individual and Mixed Lymphoid Cell Populations.*—Several additional adoptive transfer experiments employing individual and mixed lymphoid cell populations administered to lethally irradiated, syngeneic B<sub>6</sub>D<sub>2</sub>F<sub>1</sub> hybrid hosts were carried out.

The first set of experiments was performed in order to determine the differences in reconstitutive capacity of immune *versus* normal lymphoid cells in adoptive transfer of delayed responsiveness to MHSA. In these experiments,

the numbers of responder and nonresponder animals were determined at each of three differing cell dosages, by recording the number of animals per group exhibiting footpad swelling of 3.2 mm or greater. Measurements of 3.1 mm included those animals exhibiting swelling 2 SD greater than the mean of 2.7 mm for a nonsensitized group of B<sub>6</sub>D<sub>2</sub>F<sub>1</sub> hybrid mice simply challenged with

TABLE I

*The Reconstitutive Capacity of Various Individual and Mixed Lymphoid Cell Populations for Induction of Delayed Response to Methylated Human Serum Albumin in Lethally Irradiated Syngeneic Recipients*

Exp. No.	Purpose	Group No.	Status of donor of lymphoid cells*	Nature of cell inoculum		Average response footpad swelling	Number of responders	Percentage of responders
				Origin	Number†			
<i>mm</i>								
I	Reconstitutive capacity of immune vs. normal cells	A	—	—	None	2.53	—	—
		B	Normal	Spleen	10 M	3.55	6/10	60
		C	Immune	Spleen	10 M	2.65	1/10	10
		D	Normal	Spleen	20-25 M§	3.74	15/20	75
		E	Immune	Spleen	20-25 M§	3.07	7/10	35
		F	Normal	Spleen	40-50 M§	4.20	20/21	97
		G	Immune	Spleen	40-50 M§	3.39	11/22	50
II	Thymus-bone marrow cell synergism	A	Normal	Thymus	30 M	3.00	2/13	16
		B	Normal	Bone Marrow	10 M	3.10	5/13	38
		C	Normal	Thymus & bone marrow	30 M + 10 M	4.00	10/11	91
		D	—	—	None	2.53	—	—
III	Effect of additional normal bone marrow and thymus cells on reconstitution by immune or normal spleen cells	A	Normal	Spleen	20-25 M	3.74	15/20	75
		B	Immune	Spleen	20-25 M	3.07	7/20	35
		C	Normal	As per A & thymus	20 M + 30 M	3.67	10/11	91
		D	Immune	As per B & thymus	20 M + 30 M	3.66	9/9	100
		E	Normal	As per A & bone marrow	20 M + 10 M	4.11	11/12	93
		F	Immune	As per B & bone marrow	20 M + 10 M	3.70	4/8	50

\* Spleen cell donors were either immunized with goose erythrocytes 3 days previously or were normal controls of comparable age.

† Number of cells designated in millions denoted by letter M.

§ Pool of data of two separate experiments, one at each cell dose level.

MHSA. A level excluding more than 95% of a normal population was thereby designated as a cut-off point for determination of responsiveness. The background level of lethally irradiated mice either reconstituted with spleen cells or left untreated was similar to that for normal, nonirradiated animals, tending to be somewhat lower in the latter group. Thus, in these and other experiments to be described, groups of reconstituted, nonsensitized but challenged mice were omitted from the experimental protocol.

The results summarized in Table I emphasize the deficiency in reconstitution of delayed responsiveness in those animals receiving cells from mice immunized intravenously with goose erythrocytes 3 days previously, in comparison with animals receiving equal numbers of normal cells. The percentage of responders at each cell dosage was substantially lower in the immune group with a high degree of statistical significance at each of the three dosage levels employed when compared with the normal group.

If the assumption is made that the response is dependent upon one cell type, or alternatively upon one antigen-reactive cell unit occurring with a limited frequency, and furthermore, if it is also assumed that if this cell complex is present, an immune response will ensue, the frequency of the cell unit can be estimated using a Poisson distribution.

$$P(x) = \frac{\lambda^x e^{-\lambda}}{x!} \quad x = 0, 1, 2 \dots$$

The probability of a sample of nonresponders is then

$$P(0) = \frac{\lambda^0 e^{-\lambda}}{0!} = e^{-\lambda}$$

where  $\lambda = \text{No.} \times f$ , No. is total cell number, and  $f$  = frequency of the reactor cell.

A plot of  $\log P(0)$  vs No. can be used to evaluate frequency  $f$  in normal and goose-immune spleen cells. If natural logarithms are taken,

$$\begin{aligned} \log_e P(0) &= \log_e e^{-\lambda} = -\lambda \\ &= -\text{No.} \times f \end{aligned}$$

Fig. 2 illustrates the lines of best fit obtained for goose-immune and normal cells. Lines were drawn using the estimate of least square line, and goodness of fit was confirmed by using a  $\chi^2$  test.<sup>6</sup>

Estimates of antigen reactive units were:

Normal cells: 1 in  $13.5 \times 10^6$  cells, and

Immune cells: 1 in  $62.5 \times 10^6$  cells

It follows then that a goose-immune spleen cell population obtained 3 days after immunization of donor animals with 0.2 cc of 10% goose red blood cells contains 22% of reactor cells as compared with an equivalent normal spleen cell population.

The second set of experiments were carried out to evaluate the nature of the cell defect in the immune population. Consideration was given to the possi-

<sup>6</sup>  $\chi^2$  was estimated employing a simplified method described by Litchfield and Wilcoxon (*J. Pharmacol. Exp. Ther.* 1949. 96:99-113).

bility of a thymus cell-bone marrow cell interaction in transfer of delayed responsiveness, for which some evidence has been obtained in graft-*versus*-host systems (4), as well as for humoral antibody formation (8, 9). In these ex-

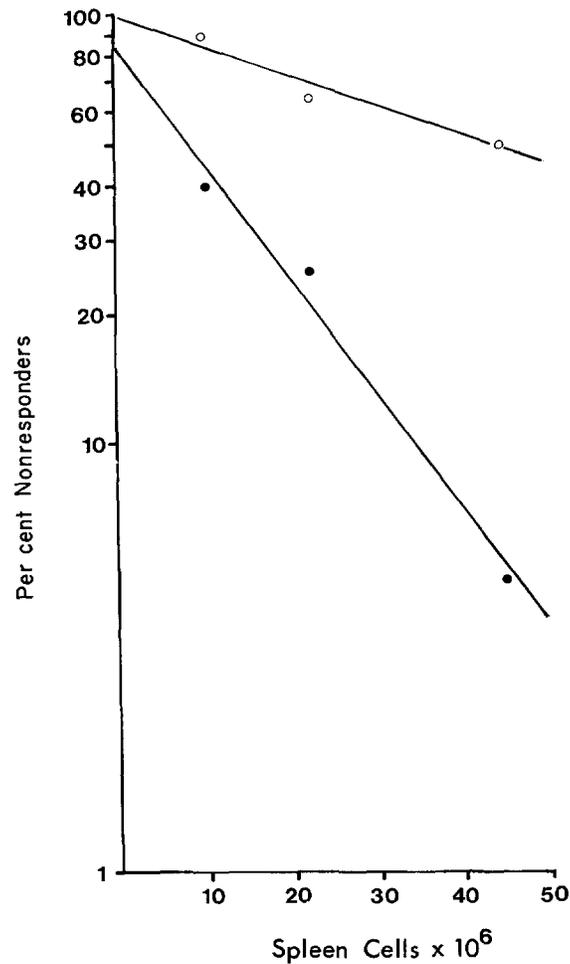


FIG. 2. Best fit lines drawn through each set of points illustrating  $\log_{10}$  percentage of nonresponders vs. spleen cell number. Mice were reconstituted with goose-immune spleen cells (open circles) or equivalent numbers of normal cells (closed circles). Experiment was carried out in  $B_6D_2F_1$  hybrid mice reconstituted with syngeneic cells (see Table I).

periments, thymus or bone marrow cells, individually as well as in a mixture, were administered to lethally irradiated, syngeneic hosts, and the response to MHSAscertained in the usual way. As indicated in Table I, a highly significant degree of difference in foodpad swelling was observed in those groups

receiving both thymus and bone marrow cells as a mixture, in comparison with the responsiveness induced by each cell population alone. Both the number of responders and the degree of response was significantly greater in the groups receiving the mixture of cells.

A third set of experiments was set up to consider the nature of the cell defect in the immune population. In order to evaluate such a defect, various cell populations were administered in combination with spleen cell populations derived from goose-immunized donors, and the reconstitutive capacity to MHSA ascertained in comparison with control animals receiving mixtures of the various lymphoid populations with normal spleen cells. As may be seen from Table I, the addition of 30 million thymus cells to 20 million immune spleen cells restored the reconstitutive capacity as measured both by the degree of footpad swelling and the number of responders. In contrast, the addition of 30 million normal thymus cells to 20 million normal spleen cells exerted little effect, if any, on the degree of footpad swelling while exhibiting equivalent numbers of responders. Furthermore, the addition of 10 million bone marrow to 20 million normal spleen cells increased the footpad swelling when compared with animals receiving normal spleen cells alone, while the addition of 10 million normal bone marrow cells to 20 million immune spleen cells did not significantly increase the number of responders, but did enhance the degree of response of the responder group.

Fig. 3 illustrates the average response and the degree of response of each member of the various experimental groups of mice. From a perusal of the scattergram of the data, the qualitative characteristics of the responses of the various groups of animals are clearly evident.

Thus, in summary, delayed response to MHSA as measured in terms of reconstitutive capacity requires the synergistic activity of a thymus and bone marrow cell population. The limiting cell in an immune population is the thymus cell, a cell which determines whether or not response occurs, while the limiting cell in the nonimmune cell population resides in a bone marrow cell component, which determines the quantitative level of response.

#### DISCUSSION

Previous data provided evidence for a cellular deficit to account for AIS, manifested by diminished humoral antibody formation to the second of a pair of antigens administered sequentially (reference 1 and footnotes 1, 5, 7). In the present work, the possibility of a cell deficit in AIS was evaluated in a model of delayed hypersensitivity. This work was performed with several further experimental purposes in mind. The observation of a cellular deficit could account for the suppression of development of delayed hypersensitivity which has been shown to be deficient in animals immunized with unrelated

<sup>7</sup> Eidinger, D., and M. Baines. 1971. Further studies of antigenic competition. II. Cellular deficit or inhibitory factor: an indirect evaluation of the problem. *Can. J. Microbiol.* In press.

antigens in a model of AIS (10-14). In view of the theory of a deficiency of thymus-bone marrow cell interaction put forth to account for suppressed humoral antibody formation in AIS, one might pose the question of whether an experimental counterpart exists for the induction of cellular immunity, namely, that cellular immunity is a consequence of thymus-bone marrow cell interaction, and whether the cellular deficit in AIS is in the one or other population. Finally, any differences in the behavior of AIS in terms of humoral antibody

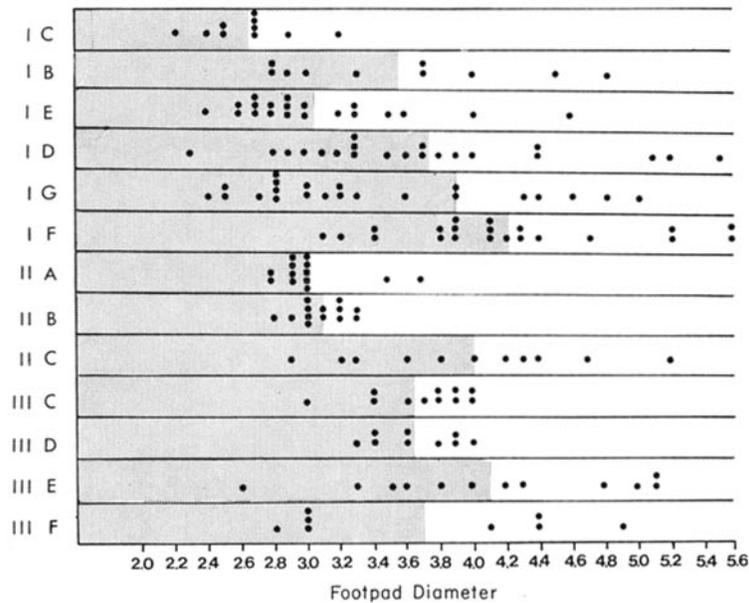


FIG. 3. A scattergram denoting footpad swelling of individual mice (each circle). Experimental details pertaining to the three illustrated groups of experiments, designated 1 B-1 G, 11 A-11 C, and 111 C-111 F are given in Table I. Demarcation of shaded area denotes the average diameter of footpad swelling.

formation as compared with cellular immunity might thus provide some insight into basic differences in the mechanisms of induction of these responses.

In the present work, an artificially prepared compound, MHSA, which has been shown to preferentially induce delayed hypersensitivity in mice (2) was chosen. The assay system makes use of the measurement of footpad swelling which has been shown to be reliable and reproducible (7).

In initial experiments, a cell transfer system was employed in order to test the capacity of a known but limited number of lymphoid cells from normal and goose-immunized animals to reconstitute delayed responsiveness to MHSA in lethally irradiated, syngeneic recipients. Both normal and goose-

immune spleen cells generated increasing numbers of responders and level of responsiveness as measured by footpad swelling, with increasing increments in cell dosage. Estimates of the frequency of antigen-reactive cells or cell units were then made employing standard statistical methods similar to those employed by Groves et al. for determining estimates of antigen-reactive cells or cell units for humoral antibody formation (15). The data indicated a frequency of 1 cell in  $13.5 \times 10^6$  cells present in a normal population, with about  $\frac{1}{4}$ – $\frac{1}{5}$  of the normal number present in a population derived from spleens of animals immunized 3 days previously with goose erythrocytes. Strong evidence that the comparative numbers are valid is indicated by a reduction in the proportion of theta-containing cells demonstrated in an equivalent 3 day goose-immune spleen cell population, in which  $\frac{1}{4}$  of the number of thymus cells was obtained in comparison with a normal population of cells (see footnote 1 and Fig. 1).

Evidence of cell synergism was obtained in experiments employing bone marrow and thymus cell populations, individually and in combination. Delayed hypersensitivity to the antigen was reconstituted in lethally irradiated recipients with nearly 100% efficiency when compared with the deficient capacities of each cell population alone to reconstitute responsiveness.

Evidence as to which of the two cell populations contains the antigen-reactive cell, and which one contains the cell analogous to the antibody-forming cell precursor (8) was forthcoming from experiments analyzing the nature of the cellular deficit in the goose-immune population, and the characteristics of this population in comparison with those of a population of normal spleen cells.

Firstly, the lack of synergism between normal spleen cells and normal thymus cells suggests that the thymus-derived cell is not lacking in a normal spleen cell population. Evidence as to its role as an antigen-reactive cell is indicated by regeneration of the reconstitutive capacity of an immune spleen cell population by the addition of thymus cells, when measured in terms of numbers of responders. Secondly, the addition of normal bone marrow cells to goose-immune spleen cells did not increase the number of responders, but greatly accentuated the level of responsiveness manifested by enhanced footpad swelling in the responder group. A similar accentuated response was obtained in animals reconstituted with mixtures of normal bone marrow and normal spleen cells.

In summary, it would appear that an immune population contains a deficient number of thymus cells which limits the numbers of animals induced to develop delayed hypersensitivity, a deficit which can be made up by the addition of normal thymus cells. Such cells are comparable to the antigen-reactive cells for humoral antibody formation. On the other hand, normal bone marrow cells are the effector cells in the reconstitution of delayed responsiveness since they are capable of enhancing the responsiveness of either an immune or

normal spleen cell population, and do not affect to a significant degree whether or not an animal is a responder.

Our findings, in the present work, are compatible with the recent findings of Hilgard (4), of Asherson and Zembala, and others (5, 16–20). These workers provided evidence for thymus–bone marrow cell synergism in the adoptive transfer of graft-*versus*-host reactivity or alternatively of cell-to-cell synergy of donor–host origin. Several groups concluded that the specificity resided with the thymus cell population, while the reactivity of the bone marrow cell was nonspecific (5, 18, 19). In the model of contact hypersensitivity employed by Asherson and Zembala (5), delayed response could be transferred by sensitized lymphocytes or alternatively, by normal macrophages which had been exposed to sensitized lymphocytes. These observations in the literature and our data derived from studies of delayed hypersensitivity to a simple modified protein, are strikingly similar in the following respects. Immunological specificity is the property of antigen acting on lymphocytes, thus altering the cells to become specifically sensitized. The capacity of transferred cells to reconstitute delayed response resides in a population of thymus-derived cells. Animals will therefore respond or not respond depending on the possession of adequate numbers of such cells. However, the effector phase of the response is dependent upon the presence of a bone marrow cell, probably the macrophage precursor, akin to the effector cell for passive transfer of intense tuberculin reactions in recipient rats (20).

The question of immune specificity resident in the bone marrow population is unresolved. The studies cited above tend to ascribe a nonspecific role to this cell population. In contrast, the failure of sensitized cells from responder guinea pigs to transfer delayed response to 2,4-dinitrophenyl-poly-L-lysine in non-responder animals suggests a specificity on a genetic basis, the nature of which remains to be elucidated (21). It is not inconceivable that the bone marrow provides two cell populations, the one being a nonspecific effector monocytic cell, and the other an immunologically specific, genetically predetermined lymphocytic cell required for induction.

The work presented herewith, in association with the current literature, provides a unified concept for reactions of delayed hypersensitivity. The model takes into account at least one biologically active factor released by reaction of antigen with sensitized lymphocytes, namely migration-inhibition factor. (a) Thymus-derived lymphocytes become sensitized by antigen or antigen fragments. The cooperative association of a bone marrow-derived lymphocyte may be needed for this step. (b) Sensitized lymphocytes release migration-inhibition factor in regions in which lymphocytes have contacted with antigen. (c) This factor then serves as a trap for macrophages derived from the bone marrow which act as the effector cells in producing delayed-hypersensitivity reactions.

The nature of the cell deficit in an immune population demonstrable in the present work, namely, deficiency in thymus-derived cells, is in agreement with the presumptive defect in immune populations for humoral antibody formation discussed in the previous papers pertaining to the underlying mechanism of AIS (antigenic competition). Immunization with unrelated antigen generating lymphoproliferation and enlarged lymphoid mass simply dilutes out the number of thymus- and/or bone marrow-derived cells required for induction of cellular immunity which is readily apparent when a fixed cell number is employed such as in cell-transfer experiments.

#### SUMMARY

A cell-transfer system was employed in the present work to investigate several characteristics of the capacity of immune and normal lymphoid cells to transfer the delayed response to methylated human serum albumin in lethally irradiated syngeneic recipients. Spleen cells derived from donor mice immunized with goose erythrocytes were far less effective in transferring responsiveness when compared with equal numbers of normal cells. Statistical analyses indicated a frequency of 1 reactive cell or cell unit in  $1.3 \times 10^7$  normal cells and in  $6.2 \times 10^7$  immune cells. These findings provided confirmatory evidence that antigen-induced suppression (antigenic competition) employing sequential administration of two non-cross-reacting antigens is due to relative deficits of immunocompetent cells generated by lymphoproliferation in lymphoid tissues secondary to immunization with the initial antigen.

The cellular deficit in the immune population was shown to be resident in a thymus cell population, which restored the number of responders to a level equivalent to the normal population. The thymic cell was akin to the antigen-reactive cell. The cell limiting the degree of response, that is the effector cell for both normal and immune cell populations, was of bone marrow origin. Both populations of cells were shown to act in synergy to reconstitute the delayed response to the antigen.

The authors acknowledge with gratitude the excellent technical assistance of Mrs. Rita Kennedy. We also wish to thank Dr. Mohan Sanduja for kindly supplying us with highly purified methanol.

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