IN VITRO INDUCTION OF SPECIFIC UNRESPONSIVENESS OF IMMUNOLOGICALLY REACTIVE, NORMAL BONE MARROW CELLS*

BY SHARWAN K. SINGHAL,† PH.D., AND HANS WIGZELL, M.D.

(From the Department of Tumor Biology, Karolinska Institute, Stockholm 60, Sweden)

(Received for publication 9 September 1969)

Recent evidence suggests that the bone marrow constitutes a major source of stem cells for humoral antibody formation (1–3). Most of the work on the importance of the bone marrow in the immune response has been analyzed by cell transfer systems in vivo. In vitro studies on normal bone marrow cells have shown that there exist in this population specific cells capable of reaction by increased DNA synthesis, if brought into contact with protein antigens (4). The immunological specificity of this cellular proliferation was demonstrated by the selective change in reactivity to antigens of bone marrow cells derived from immune or immunologically tolerant animals (5). Density gradient centrifugation yielded information suggesting that the antigen-reactive cell of the normal bone marrow had the morphological characteristics of a small lymphocyte (6).

In the present article we present further information on the existence and behavior of preformed, antigen-reactive cells in the population of normal bone marrow cells. Glass bead columns coated with antigenic molecules can be utilized as immunological filters for cell populations containing immunologically reactive cells of relevant specificity (7). Specific fractionation of cells reactive against the antigen used for coating the column was obtained by passage through that column, as analyzed by the subsequent study of the antibody-producing capacity of the fractionated cells in vitro or in vivo (7). A preliminary report that normal bone marrow cells can be separated on antigen-coated columns has been published (8) and confirmed (9). The present article contains detailed information on the existence of preformed antigen-specific surface receptors on antigen-reactive cells in normal bone marrow, as analyzed by immunological filtration studies.

Controversy exists as to whether immunological tolerance resides in the thymus-derived immune cells (1, 10, 11) and/or whether paralysis can also be demonstrated in bone marrow–derived immunocytes (12). The present in vitro

* This work was supported by the Swedish Cancer Society, Carl-Bertil Nathorsts Vetenskapliga och Allmännyttiga Stiftelser, the Jane Coffin Childs Memorial Fund for Medical Research, Ulla och Karl Svanlunds Stiftelse and the Medical Research Council of Canada.

† Fellow of the Medical Research Council, Canada.
system lends itself well to studies of immunological tolerance. Induction of immunological unresponsiveness towards a given antigen could be demonstrated by cultivation of normal bone marrow cells together with immunogen of varying concentration. Dose-response curves for the induction of specific DNA synthesis by immunogen in vitro were calculated. The theoretical implications of the above results will be analyzed and discussed.

Materials and Methods

Animals.—Adult, 3-4 lb., white outbred rabbits were used throughout these experiments.

Biologicals.—The antigens used were HSA\(^1\) (human serum albumin, Kabi AB, Stockholm, Sweden), BSA (bovine serum albumin, fraction V, Armour and Co., Eastbourne, England), and OA (ovalbumin, Worthington Biochemical Corp., Freehold, N. J.). All antigens were dissolved in Medium 199 (State Bacteriological Laboratories, Stockholm) and sterilized through Millipore filter membranes before use. NRS (normal rabbit serum) was collected by bleeding the bone marrow donor by cardiac puncture prior to killing the rabbit.

Beads and Columns.—The experimental procedure and theoretical implications of antigen coated glass bead columns have been described at length in a previous communication from this laboratory (7). Briefly, glass beads of an average diameter of 200 μ (Superbrite, 100-5005, Minnesota Mining & Manufacturing Co., St. Paul, Minn.) were used. The beads were first boiled in aqua regia for 15 min, then washed in phosphate-buffered sterile saline until a pH of 7.4 was obtained. Antigen at a concentration of 5 mg/ml was added to the fluid containing beads and incubated at 45°C for 1 hr, followed by overnight incubation at 4°C. Subsequently, the beads were allowed to stand at room temperature in 10% normal rabbit serum for 1 hr, a procedure which allows less mechanical retention of passing cells (7). 1.5 × 100 cm glass columns were sterilized by incubation with 70% ethanol, then rinsed with sterile saline. The beads were poured into the columns and approximately 200 ml of saline, followed by 100 ml of medium 199 were allowed to pass through the bead column to remove unattached free antigen. The columns were cooled to 4°C and the bone marrow cells to be tested were applied to the top of the column. The rate of passing fluid through the column was kept at approximately 2 ml/min. The passed cells were collected in sterile tubes. The cells retained by the column were recovered by removing the bottom of the column and letting the beads drop into a sterile bottle containing 30-50 ml of medium. The beads were gently shaken a few times with round wrist movement and the supernatant containing the retained cells was removed. All cells were washed twice with cold medium before analysis in tissue culture for their capacity to respond to relevant antigens. Gentle shaking when removing the retained cells by mechanical means is critical, since great cell death would otherwise occur.

Tissue Culture.—Medium 199 containing 10% NRS and 100 μg streptomycin and 100 units penicillin per ml was used. Rabbits were sacrificed by an intravenous administration of 10 ml of air. The bone marrow cells were collected by flushing the tibia and femur with a syringe which contained medium. The cell mass was shaken, filtered through a sterile 50-mesh wire net to remove large particles of fat and was then centrifuged at 600 g for 8 min. The thin fatty layer and the supernatant were decanted, the cells suspended in medium, and kept at 4°C throughout until cultured. The appropriate cell suspensions were prepared to contain 10⁶ cells per ml and in 4 ml volumes, distributed into sterile plastic tubes (Falcon Plastics, Los Angeles, Calif.). Appropriate amounts of antigen(s) in 0.1 ml volumes were added, the tubes sealed, and kept at 37°C for 4 days. Approximately 18 hr before conclusion of the experi-

\(^1\)Abbreviations used in this paper: BSA, bovine serum albumin, fraction V; cpm, counts per minute; HSA, human serum albumin; NRS, normal rabbit serum; OA, ovalbumin.
ment, 2 μc of tritiated thymidine (1.0 c/mmole, New England Nuclear Corp., Boston, Mass.) was added to each tube. At termination, the tubes were centrifuged at 1500 g for 10 min, the supernatants discarded, and the cell mass washed twice with 2 ml of 5% trichloroacetic acid. 0.5 ml of Hyamine (dimethylbenzylammonium hydroxide, Packard Instrument Co., Inc., Downers Grove, Ill.) was added to each tube and kept overnight at room temperature in complete darkness. The contents of the tubes were transferred to Packard counting vials containing 15 ml of scintillation fluid (15.2 g 2,5-diphenyloxazole (PPO), 0.380 g 1,4-bis-2-(5-phenyloxazolyl)benzene (POPOP), 40% Triton X-100, made up to 3.8 liters with toluene). The vials were cooled to 4°C in dark and analyzed for radioactivity by a Packard liquid scintillation counter model 500D.

| Cells          | Uptake of tritiated thymidine (cpm) | HSA* | OA* |
|----------------|--------------------------------------|------|-----|
|                |                                       | 1.25 mg/ml | 0.25 mg/ml |
| Control        | 3.612 ± 0.029                        | 3.896 ± 0.066 | 3.950 ± 0.046 |
| Passed         | 3.116 ± 0.448                        | 4.034 ± 0.035 | 4.100 ± 0.021 |
| Retained       | 3.652 ± 0.040                        | 4.157 ± 0.014 | 4.183 ± 0.025 |
| Passed         | 3.510 ± 0.027                        | 3.934 ± 0.100 | 4.213 ± 0.040 |
| Retained       | 4.218 ± 0.032                        | 4.457 ± 0.009 | 4.604 ± 0.056 |
| Passed         | 4.3512 ± 0.055                       | 4.0004 ± 0.016 | 3.8587 ± 0.056 |
| Retained       | 4.3443 ± 0.033                       | 4.5919 ± 0.024 | 4.6895 ± 0.020 |

* Figures expressed in log10 values. Mean ± standard error of the mean. Each group contains four tubes. Three different, normal serum-coated columns were run in parallel.

RESULTS

Distribution Pattern of Normal, Antigen-Reactive Cells of the Bone Marrow on Bead Columns Coated with Normal Serum Only

It has previously been found that high-rate antibody forming cells as well as immunological memory cells (7) display a slightly enhanced capacity to become retained in a serologically nonspecific fashion when filtered through columns coated with normal serum only. In the present system, normal rabbit bone marrow cells were fractionated on bead columns coated with the serum of the bone marrow donor, in order to exclude any possible separation due to foreign serum protein antigens. The control, the passed, and the mechanically eluted, retained cells were subsequently analyzed for their sensitivity to become stimulated into increased DNA synthesis in vitro. The results are shown in Table I and demonstrate that the mechanically retained cells have a significantly increased DNA synthesis in the absence of antigen stimulation,
compared to the control and passed cells. We believe this to be caused by the
damage inflicted upon the retained cells through mechanical elution, as merely
shaking the control cell population with the beads, in the same way, to obtain
"retained" cells could be shown to significantly increase DNA synthesis (Table
II). Despite the increase in DNA synthesis in the absence of antigenic stimula-
tion, there was no difference in the degree of stimulation with antigen in the
three different cell groups, indicating that the specific increase of DNA syn-
thesis by antigen was undisturbed and superimposed on the background DNA
synthesis. We found that there exists a positive correlation between the back-
ground DNA synthesis of the untreated cells and their capacity to become
stimulated by antigen into an enhanced DNA synthesis. This correlation could

| Antigen          | Uptake of tritiated thymidine (cpm) |
|------------------|-------------------------------------|
|                  | Normal cells*                        | Cells shaken with glass beads* |
| Unstimulated     | 3.8317 ± 0.015                      | 4.3792 ± 0.025                |
| BSA 1.25 mg/ml   | 4.5380 ± 0.049                      | 4.8106 ± 0.021                |
| BSA 0.25 mg/ml   | 4.2786 ± 0.037                      | 4.7053 ± 0.010                |
| BSA 0.025 mg/ml  | 3.8092 ± 0.069                      | 4.5535 ± 0.064                |

* Figures expressed in log10 values and as mean ± standard error of the mean. Each group
contains four tubes.

be transformed into a constant factor valid for samples from a given cell popu-
lation stimulated with the same antigen if the values for DNA synthesis were
expressed in log10 values. The ability of a given cell suspension to be stimulated
by using a certain antigen would then be expressed as the difference between
the DNA synthesis in the stimulated population as compared to that of the
untreated cells (log10 mean of the stimulated group minus log10 mean of the
control group). This method of calculating reactivity to antigenic stimulation
in vitro has been followed throughout this article.

Specific Fractionation of Normal Antigen Reactive Bone Marrow
Cells by Antigen Coated Columns

Normal bone marrow cells were applied to two separate columns coated either with HSA
or OA. Passed and retained cells were recovered as described in the previous chapter and
cultured in vitro in the presence or absence of HSA or OA. The unpassed cells were always
cultured in the same way to serve as controls. The results are presented in Table III.
The experiments yield conclusive and clearcut results. Cells that had passed through an antigen-coated column display a selective decrease in reactivity towards that antigen in vitro whereas the retained, mechanically eluted cells show a specific increase towards the same antigen. A total of 31 experiments were carried out, of which 26 yielded results showing a significant change of

### TABLE III

**Antigen-Induced DNA Synthesis of Normal Bone Marrow Cells Separated on Antigen-Coated Columns**

| Exp. | Column | Cells    | Uptake of tritiated thymidine (cpm) | HSA/OA | Relative reactivity |
|------|--------|----------|-------------------------------------|--------|--------------------|
|      |        |          | 1.25 mg/ml | 0.25 mg/ml |                   |
| 1    | Control|          | 4.6581 | 5.0598 | 5.1735 | 0.8863 | -1 | 100 |
|      | HSA    | Passed   | ±0.037 | ±0.051 | ±0.049 | 0.7937 | -1 | 81  |
|      |        | Retained | ±0.016 | ±0.034 | ±0.025 | 0.4790 | 391 |
|      | OA     | Passed   | 4.8370 | 4.9907 | 5.1970 | 0.1091 | 167 |
|      |        | Retained | ±0.046 | ±0.005 | ±0.053 | 0.6528 | -1 | 58  |

* Figures expressed in log₁₀ values. Mean ± standard error of the mean. Each group contains four tubes.

** Relative reactivity, HSA/OA values of different groups as compared to control ratio (control ratio = 100).
distribution pattern of specific reactivity after filtration of cells through an antigen-coated column. In the remaining negative experiments, the results were considered as suggestive of specific separation but values of significance were low ($P > 0.1$). No experiment yielded results contrary to those reported above.

As can be seen in Table III, a significant increase in the background DNA synthesis was found in all retained cell populations. The degree of enhanced thymidine incorporation as compared to control and/or passed cell populations was similar, as found previously when using columns coated with normal rabbit serum only (Table IV). We consider that this increase was a consequence of the mechanical elution procedure and not caused by the bead-attached antigen.

**TABLE IV**

DNA Synthesis of Unstimulated Bone Marrow Cells Fractionated on Antigen-Coated or Normal Serum-Coated Columns

| Column       | Unstimulated* cells | Passed* cells | Unstimulated*/ Passed* | Retained* | Unstimulated*/ Retained* | No. of columns |
|--------------|----------------------|--------------|------------------------|-----------|--------------------------|----------------|
| Antigen      | 3.8807               | 3.7904       | 0.0851                 | 4.3308    | 0.5830                   | 17             |
| Serum        | ±0.160               | ±0.164       | ±0.118                 | ±0.119    | ±0.101                   |                |
| Normal serum | 3.6127               | 3.3859       | 0.2268                 | 4.1066    | 0.5061                   | 4              |
| Serum        | ±0.029               | ±0.133       | ±0.112                 | ±0.213    | ±0.136                   |                |

* Figures expressed in log10 values. Mean of the means of each experiment ± standard error of the mean.
† 6 HSA-coated, 5 BSA-coated and 6 OA-coated columns.

The Effect of Free Antigen on the Separation of Normal Antigen-Sensitive Cells by the Antigen-Coated Columns

This experiment was designed in accordance with the postulate of a cell-associated antibody where the presence of free antigenic molecules in the columnar fluid during the cellular filtration would be expected to compete with the bead-attached antigen for cellular "antibody", thus blocking the elimination of specific antigen-reactive cells. This has previously been shown to function when testing high-rate antibody-forming and memory cells passing antigenic columns (7).

Normal bone marrow cells were incubated with or without free antigen for 5 min, whereafter the cells were applied to antigen-coated columns in the presence or absence of free antigen in the columnar fluid. The concentration of free antigen was kept between 0.5 to 2 mg/ml of fluid which has been shown to function in blocking the selective elimination of plaque-forming cells (PFC) or memory cells passing through antigenic columns (7). After the fractionation, all cells were washed twice with cold medium, counted, and assayed in vitro for their capacity to undergo DNA synthesis in the presence of HSA or OA.

The results in Table V show a specific blocking of separation by the use of free antigen in the column, whereas specific separation was achieved by the
columns which contained no free antigen. This experiment further substantiates the concept of the "antibody" receptors on the outer surface of the normal antigen-reactive cells present in the bone marrow.

The Response to Antigen of Normal Antigen-Reactive Cells when Applied but not Allowed to Pass Completely through the Antigen-Coated Columns

To study further the specificity of the normal antigen-reactive cells and function of the antigen-coated columns, normal bone marrow cells were applied to two separate columns, each coated with BSA. In one column the cells were allowed to pass through and both the passed and retained fraction were recovered as described earlier. The cells applied to the second column were allowed to travel to the bottom end, whereupon the column beads were recovered and the "passed and retained" fraction eluted, cultured, and compared with the passed and retained cells of the first column and the cells that were not applied to the column.

The results are presented in Table VI and show that the "passed and retained" cells behave in a similar fashion as the control cells which were not applied to the column. On the other hand, a specific separation was accomplished in the first column, where the cells were allowed to pass through completely. These results further strengthen the concept that the antigen-coated column merely serves as an immunological filter without activating the specific antigen-reactive cells, as the passed and retained cells could be shown to have the same immunological reactivity as the original cell suspension.

Induction of Specific Immunological Unresponsiveness of Normal Bone Marrow Cells In Vitro

Controversy exists as to whether specific unresponsiveness can be induced in antigen-reactive cells derived from the bone marrow (1, 10, 11). As the immunological nature of the DNA stimulation of normal bone marrow cells in vitro by antigen is well established, (4, 5, and the present article) we decided to investigate whether immunological paralysis could be demonstrated in the present system.

The experimental set-up consisted of cultivating samples of the same batch of normal bone marrow in two series of tubes. To both series a varying concentration of one antigen was added, whereas a constant dose of a second antigen was added to only one series of tubes. The concentration of the second antigen was chosen as to induce optimal DNA stimulation of the normal bone marrow cells. Induction of paralysis against the first antigen under certain conditions could then be verified to be immunological in nature if a normal response towards the second antigen could be demonstrated in the parallel group of tubes.

The results shown in Table VII and Figs. 1-3, clearly demonstrate a specific lack of DNA stimulation when too high a concentration of one antigen is used. That this lack of increase of thymidine uptake by these cells is immunological and not caused by unspecific toxicity of the high amount of antigen is shown by the normal DNA stimulation by a second antigen in optimal concentration. It
### Table V

**Antigen-Induced DNA Synthesis of Normal Bone Marrow Cells Separated on Antigen-Coated Columns Containing Extra Free Antigen**

| Exp. | Column | Cells    | Free antigen | Uptake of tritiated thymidine (cpm) | HSA/OA | Relative reactivity |
|------|--------|----------|--------------|-------------------------------------|--------|---------------------|
|      |        |          |              | Unstimulated*                        | OA*    |                     |
|      |        |          |              | 1.25 mg/ml                           | 0.25 mg/ml |                     |
| 1    | Control | ---      | ---          | 3.5497 ± 0.003                        | 3.8551 ± 0.053 | 3.9956 ± 0.094 | 0.9195 | 100 |
|      | HSA    | "        | HSA          | 3.5973 ± 0.033                        | 3.6891 ± 0.007 | 3.9063 ± 0.060 | 0.7828 | 100 |
|      | Passed  | "        | HSA          | 2.7795 ± 0.068                        | 3.2537 ± 0.117 | 3.5884 ± 0.134 | 0.6653 | 56 |
|      | Retained| "        |              | 4.1775 ± 0.019                        | 4.4418 ± 0.050 | 3.9296 ± 0.056 | 0.5122 | 391 |
|      | Passed  | HSA      | HSA          | 2.5554 ± 0.095                        | 3.0954 ± 0.044 | 3.1825 ± 0.112 | 0.9129 | 135 |
|      | Retained| "        |              | 4.2266 ± 0.027                        | 4.3305 ± 0.056 | 4.1630 ± 0.036 | 0.1674 | 242 |

| Passed/Retained | HSA column = 0.1531 | Free HSA column = 0.7454 |
|-----------------|---------------------|-------------------------|
| 2    | Control | ---      | ---          | 4.0410 ± 0.025                        | 4.4698 ± 0.036 | 4.47189 ± 0.0022 | 0.7509 | 100 |
|      | HSA    | "        | HSA          | 3.8680 ± 0.039                        | 3.9894 ± 0.025 | 4.3884 ± 0.049 | 0.6010 | 100 |
|      | Passed  | "        | HSA          | 3.8386 ± 0.039                        | 4.0495 ± 0.053 | 4.5526 ± 0.043 | 0.4969 | 56 |
|      | Retained| "        |              | 4.5133 ± 0.063                        | 4.9144 ± 0.035 | 4.6896 ± 0.026 | 0.2248 | 298 |
|      | Passed  | HSA      | HSA          | 3.1535 ± 0.089                        | 3.6506 ± 0.115 | 4.1030 ± 0.090 | 0.5476 | 88 |
|      | Retained| "        |              | 3.2522 ± 0.072                        | 3.7783 ± 0.079 | 4.0379 ± 0.054 | 0.7406 | 138 |

* Figures expressed in log10 values. Mean ± standard error of the mean. Each group contains four tubes.

† Relative reactivity, HSA/OA values of different groups as compared to control ratio (control ratio = 100). Two controls, with or without free HSA.
### Table VI

**Antigen-Induced DNA Synthesis of Normal Bone Marrow Cells Applied but not Allowed to Pass Completely Through Antigen Coated Columns**

| Exp. | Column | Cells     | Uptake of tritiated thymidine (cpm) | BSA*       | OA*       | BSA*/OA | Relative † reactivity |
|------|--------|-----------|-------------------------------------|------------|-----------|----------|-----------------------|
|      |        |           |                                     | 0.25 mg/ml | 0.05 mg/ml |          |                       |
| 1    | —      | Control   |                                     | 3.6526 ± 0.017 | 4.1412 ± 0.095 | 4.1979 ± 0.038 | 0.9433 | -1 | 100 |
|      |        | BSA Passed |                                     | 4.5371 ± 0.016 | 4.4651 ± 0.012 | 4.8537 ± 0.017 | 0.6114 | -1 | 47 |
|      |        | Retained  |                                     | 3.4689 ± 0.066 | 3.8939 ± 0.027 | 3.3999 ± 0.071 | 0.4940 |       | 355 |
|      |        | Passed and retained |                     | 3.3945 ± 0.036 | 3.8633 ± 0.027 | 3.9729 ± 0.013 | 0.8904 | -1 | 89 |
|      | Control BSA/OA | = 0.3319 |                                     |            |            |          |                       |
|      | Passed BSA/OA | = 0.4503 |                                     |            |            |          |                       |
|      | Retained BSA/OA | = 0.0529 |                                     |            |            |          |                       |
| 2    | —      | Control   |                                     | 3.5061 ± 0.044 | 3.9660 ± 0.043 | 3.9964 ± 0.059 | 0.9696 | -1 | 100 |
|      |        | BSA Passed |                                     | 3.6242 ± 0.050 | 3.8099 ± 0.065 | 4.2386 ± 0.011 | 0.5713 | -1 | 40 |
|      |        | Retained  |                                     | 3.8793 ± 0.003 | 4.5302 ± 0.026 | 3.8752 ± 0.062 | 0.6550 |       | 485 |
|      |        | Passed and retained |                     | 3.7472 ± 0.016 | 4.1196 ± 0.051 | 4.1483 ± 0.046 | 0.9713 | -1 | 100 |
|      | Control BSA/OA | = 0.3983 |                                     |            |            |          |                       |
|      | Passed BSA/OA | = 0.3146 |                                     |            |            |          |                       |
|      | Retained BSA/OA | = 0.9983 |                                     |            |            |          |                       |

* Figures expressed in log, values. Mean ± standard error of the mean. Each group contains four tubes.

† Relative reactivity, BSA/OA values of different groups as compared to control ratio (control ratio = 100).
### Table VII

*Induction of Specific Unresponsiveness In Vitro of Normal Bone Marrow Cells by Incubation with Varying Concentrations of Antigen. Control of Specificity: Stimulation with an Optimal Dose of a Second Antigen*

| Exp. | Varying antigen | Constant antigen* | Antigen concentration of the varying antigen (mg/ml)† |
|------|----------------|-------------------|---------------------------------------------------|
| 1    | OA             | —                 | 15 × 10⁻⁴  | 60 × 10⁻⁴  | 25 × 10⁻⁴  | 10⁻³       | 5 × 10⁻⁶  | 10⁻⁹  | 5 × 10⁻¹² |
|      | OA             | HSA               | 3.741 ± 0.02 | 3.890 ± 0.09 | 4.344 ± 0.04 | 4.192 ± 0.05 | 4.074 ± 0.02 | 3.755 ± 0.03 | 3.726 ± 0.01 |
|      | OA             | HSA               | 4.232 ± 0.03 | 4.226 ± 0.03 | 4.230 ± 0.03 | 4.418 ± 0.04 | 4.306 ± 0.02 | 4.240 ± 0.02 | 4.081 ± 0.03 |
|      | HSA            | —                 | 3.759 ± 0.04 | 3.753 ± 0.01 | 3.890 ± 0.05 | 4.126 ± 0.01 | 4.366 ± 0.04 | 4.300 ± 0.02 | 3.865 ± 0.02 |
|      | HSA            | OA                | 4.177 ± 0.03 | 4.357 ± 0.05 | 4.214 ± 0.01 | 4.418 ± 0.02 | 4.286 ± 0.02 | 4.040 ± 0.03 | 4.150 ± 0.03 |
| 2    | BSA            | —                 | 3.757 ± 0.03 | 3.779 ± 0.04 | 4.199 ± 0.03 | 4.600 ± 0.01 | 4.882 ± 0.01 | 4.771 ± 0.01 | 3.909 ± 0.05 |
|      | BSA            | OA                | 4.534 ± 0.02 | 4.454 ± 0.02 | 4.577 ± 0.02 | 4.566 ± 0.05 | 4.901 ± 0.01 | 4.786 ± 0.01 | 4.598 ± 0.07 |
| 3    | BSA            | —                 | 4.285 ± 0.03 | 4.289 ± 0.04 | 4.443 ± 0.02 | 4.918 ± 0.04 | 4.991 ± 0.06 | 4.618 ± 0.05 | 4.313 ± 0.04 |
|      | BSA            | OA                | 4.965 ± 0.01 | 5.025 ± 0.01 | 4.984 ± 0.03 | 4.956 ± 0.03 | 4.965 ± 0.02 | 4.912 ± 0.02 | 4.846 ± 0.06 |

* Concentration of constant antigen: OA 0.25 mg/ml, HSA 1.25 mg/ml.
† DNA synthesis expressed in log₁₀ values of cpm. Top figures of results denote mean ± standard error of the mean. Figures within brackets depict per cent reactivity of the peak values for the varying antigen as compared to the untreated control. Figures within brackets when using the constant antigen depict per cent reactivity as compared to constant antigen only.
Fig. 1. Dose response curve of DNA synthesis induced by HSA. Impact of the addition of a second antigen, OA, in optimal concentration. DNA synthesis expressed in log_{10} values of cpm ± standard error of the mean.

Fig. 2. Dose response curve of DNA synthesis induced by OA. Impact of the addition of a second antigen, HSA, in optimal concentration. DNA synthesis expressed in log_{10} values of cpm ± standard error of the mean.
can also be seen from the results in Table VII and Figs. 1–3 that cultivation with a mixture of two antigens under optimal conditions for both antigens does not add any significant DNA stimulation to that obtained by one antigen only. The finding that OA will stimulate DNA uptake at a lower concentration than HSA or BSA is in agreement with the greater immunogenicity of OA in vivo as expressed by the induction of immunity with lower doses of immunogen.²

These results support the concept that bone marrow-derived antigen-reactive cells can be rendered specifically and immunologically unresponsive.

![Dose response curve of DNA synthesis induced by BSA. Impact of the addition of a second antigen, OA, in optimal concentration. DNA synthesis expressed in log_{10} values of cpm ± standard error of the mean.](image)

**DISCUSSION**

Accumulating evidence strongly suggests that complex cellular interactions are necessary for induction and the appearance of high-rate antibody-forming cells (1, 2, 13). Cellular cooperation has been demonstrated to take place between lymphoid cells derived from different organ systems, such as thymus and bone marrow (1–3), and to involve intermarriage between cells reacting against different antigenic sites present on the same immunogenic molecule (13, 14). Precursors for high-rate antibody synthesis have been shown in the mouse to involve cells of bone marrow origin (1, 2), whereas thymus-derived lymphocytes are capable of enhancing the antibody-forming capacity

² Andersson, B., and H. Wigzell. 1969. Unpublished data.
of the bone marrow-derived cells (1–3, 10, 11). In the rabbit, normal bone marrow cells contain antigen-reactive elements as indicated by induction of DNA synthesis by antigen in vitro (4–6, 8, and the present article). The immunological specificity of this reactivity is certified by the selective change in antigenic reactivity of bone marrow cells taken from early immune or immunologically tolerant animals, where in both systems a selective decrease in reactivity towards stimulation with the relevant antigen was recorded (5). The mechanism(s) underlying this diminished reactivity after introduction of immunogen and/or tolerogen in vivo is not well understood. In the normal rabbit, bone marrow cells are unique in their response to antigenic stimulation in vitro, as no such response was ever recorded with cells derived from normal lymph node, spleen, or thymus (4). This distribution pattern of reactivity is not limited to the rabbit, however, but could also be shown to occur in another animal species tested, the mouse (17). In view of the importance of the bone marrow system for the provision of potential, high-rate antibody-forming cells (1–3) further studies on the behavior of isolated antigen-reactive cells of the normal bone marrow in vitro might yield important information on their role in the complex building up of an immune response. It remains, however, to be established whether the singular reactivity in vitro of the normal bone marrow cells is due to the existence of a specific, antigen-recognizing cell unique for the marrow, or whether there exist marrow-specific cells capable of responding in a secondary manner by increased DNA synthesis as a consequence of a reaction in their vicinity between antigen and an antigen-recognizing cell. A selective elimination from the passed population of cells reactive against the antigen used for coating the column has previously been reported in detail (7), whereas only very preliminary reports on the elimination of the immune reactivity of normal spleen, lymph node, (15) or bone marrow cells (8) have been presented from this laboratory.

In the present study we could show that normal bone marrow cells have preformed antibodies on their outer surface, allowing a selective retention of the relevant antigen-reactive cells when filtered through antigen-coated columns. The test for the immune potential of the separated bone marrow cells involved stimulation with antigens in vitro and measure of DNA synthesis. Blocking of the specific eliminatory capacity of the column was obtained with free antigen in the medium during cellular filtration, in agreement with the surface antibody concept. The blocking argues against the possibility that immunological tolerance was induced in the passing cells by the bead-attached antigen, as a normal DNA response was delivered by these cells. In some experiments with no free antigen in the medium, cells were allowed to pass through to the bottom of the column, whereupon all cells recovered were cultured together in vitro (passed and retained cells) with antigen.

No change in immunological reactivity towards antigenic stimulation with the antigen used for coating the column or with a second antigen was observed in these experiments, indicating that the cellular attachment to the bead-linked antigen for a short time in vitro did not cause any change in the immunological reactivity of the cells. A substantial percentage of the bone
marrow cells filtering through the columns was retained (around 50% of the cells applied to the column), mainly due to non-immunological forces, as similar retention figures were obtained when using columns coated with normal serum only. These high numbers would tend to largely disqualify the bead columns for enrichment of antigen reactive cells, as the absolute immune reactivity per unit cell number of the retained cells when tested against the antigen used for coating the column was only slightly enhanced (less than by a factor of 2) when compared to the reactivity of the control cells.

In a very recent article, however, results have been presented on the separation of antigen-sensitive normal rabbit bone marrow cells on antigen-coated columns followed by an analysis in vivo of their immune potential after transfer into allogeneic rabbits (9). In disagreement with our results (7, 8, 15, and the present article), they report a close to 50-fold absolute increase in specific immune reactivity of the retained cells, indicating not only a passive enrichment of the administered antigen-reactive cells by the column, but also a significant specific stimulation of the immune reactivity of the column-administered cells by the column antigen (9). This finding, in conjunction with the exceedingly narrow range of variation in the number of antibody-forming cells in their system (Table I, Ref. 16), would suggest that their cellular system involves certain unique features.

As mentioned in the introduction, conflicting views exist as to the possibility of inducing immunological paralysis in bone marrow-derived lymphocytes (1, 10–12). In the present system we could demonstrate that there exists a clear-cut dose-response relationship between the antigen dose and the subsequent DNA response of the marrow cells. Supraoptimal concentration of one antigen failed to induce increased DNA synthesis and the immunological nature of this lack of reactivity was indicated by the normal response of the same cells when confronted with an optimal dose of a second antigen. The molecular concentration of three different antigens needed to induce this state of specific unresponsiveness was found to be similar, whereas differences were recorded when finding the lowest concentration of antigen capable of inducing a maximum increase in DNA synthesis above background values. There exists a positive correlation between the lowest dose of an antigen needed to induce detectable immunity in vivo (13) and that necessary to increase the DNA synthesis of normal bone marrow cells in vitro. No evidence was found of a two-zone dose phenomenon in the present induction of a state of specific unresponsiveness.

We consider the above data in support of the view that bone-marrow derived lymphocytes can be rendered immunologically tolerant (12). We would like to stress, however, that caution should be applied in the interpretation of the present results. Specific lack of reactivity towards antigenic stimulation in vitro has been demonstrated, but it remains to be established as to what consequences this lack of reactivity would have for the potential capacity of the
marrow population to produce precursor cells for high-rate antibody formation (1-3).

In conclusion, normal bone marrow cells could be separated according to specific antigenic reactivity by allowing the cells to sieve through antigen-coated bead columns. Experimental evidence supported the view that the fractionation was caused by the existence of preformed, antigen-reactive receptors present on the outer surface of the antigen-reactive cells. It is assumed that these receptors play a vital role in the recognition of antigen, but formal proof for this is lacking. Specific paralysis of the antigen-reactive cells of the normal bone marrow could be induced by antigen in vitro as indicated by the absence of increased DNA synthesis.

SUMMARY

Normal rabbit bone marrow cells have been studied according to their immunological reactivity in vitro. The test system involved stimulation by antigen after the subsequent stimulation into cellular proliferation by measuring the uptake of tritium-labeled thymidine. Specific separation of immunological reactivity was obtained by filtration of cells through antigen-coated bead columns. All experimental evidence supported the view that this separation was due to the existence of preformed antibody molecules on the outer cell surface of the antigen-recognizing cells.

The response to antigenic stimulation was shown to be strictly dose related and, using supraoptimal concentrations of one antigen, no increased DNA synthesis was recorded. That this state of unresponsiveness represented a state of immunological paralysis was indicated by the normal response of these cells to stimulation by a second antigen in optimal concentration.

Thus both methods, cell separation on antigen-coated columns or induction of specific unresponsiveness by antigen in vitro, can produce a cell population specifically devoid of cells reactive against a given antigen.

The excellent technical assistance of Miss Ulla Källström and Miss Margareta Svänesson is gratefully acknowledged.

BIBLIOGRAPHY

1. Miller, J. F. A. P., and G. E. Mitchell. 1968. Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. J. Exp. Med. 128:801.
2. Claman, H. N., E. A. Chaperon, and R. F. Triplett. 1966. Immunocompetence of transferred thymus-marrow cell combinations. J. Immunol. 97:828.
3. Davies, A. J. S., E. Leuchars, V. Wallis, R. Marchand, and E. V. Elliot. 1967. The failure of thymus derived cells to produce antibody. Transplantation. 6:222.
4. Singhal, S. K., and M. Richter. 1968. Cells involved in the immune response. I. The response of normal rabbit bone marrow cells to antigens in vitro. Int. Arch. Allergy Appl. Immunol. 33:493.
5. Singhal, S. K., and M. Richter. 1968. Cells involved in the immune response. IV. The response of normal and immune rabbit bone marrow and lymphoid tissue lymphocytes to antigens in vitro. *J. Exp. Med.* **128**:1099.

6. Singhal, S. K., M. Richter, and D. G. Osmond. 1968. Cells involved in the immune response. III. Responsiveness to antigens of lymphocyte-rich fraction of normal rabbit bone marrow. *Int. Arch. Allergy Appl. Immunol.* **34**:224.

7. Wigzell, H., and B. Andersson. 1969. Cell separation on antigen-coated columns. Elimination of high rate antibody forming cells and immunological memory cells. *J. Exp. Med.* **129**:23.

8. Singhal, S. K., and H. Wigzell. 1969. Antigen specific receptors on the outer cell surface of "virgin" immunocytes of the normal bone marrow. *Fed. Proc.* **28**:309.

9. Abdou, N. I., and M. Richter. 1969. Cells involved in the immune response. X. The transfer of antibody-forming capacity to irradiated rabbits by antigen-reactive cells isolated from normal allogeneic rabbit bone marrow after passage through antigen-sensitized glass bead columns. *J. Exp. Med.* **130**:141.

10. Taylor, R. B. 1968. Immune paralysis of thymus cells by bovine serum albumin. *Nature (London).* **9.20**:611.

11. Taylor, R. B. 1969. Cellular cooperation in antibody response of mice to two serum albumins: Specific function of thymus cells. *Transplant. Rev.* **1**:114.

12. Playfair, J. H. L. 1969. Specific tolerance to sheep erythrocytes in mouse bone marrow cells. *Nature (London).* **222**:883.

13. Mitchison, N. A. 1967. Antigen recognition responsible for the induction in vitro of the secondary response. *Cold Spring Harb. Symp. Quant. Biol.* **32**:731.

14. Rajewsky, K. V., S. Schirmacher, S. Nase, and N. K. Jerne. 1969. The requirement of more than one antigenic determinant for immunogenicity. *J. Exp. Med.* **129**:1131.

15. Wigzell, H. 1969. Use of humoral or cell bound antibody to achieve depression of immunological reactivity. *Antibiot. Chemother.* **15**:82.

16. Richter, M., and N. I. Abdou. 1969. Cells involved in the immune response. VII. The demonstration, using allotypic markers, of antibody formation by radioresistant cells of irradiated rabbits injected with normal allogeneic bone marrow cells and sheep erythrocytes. *J. Exp. Med.* **129**:1261.

17. Singhal, S. K., and H. Wigzell. 1970. Cognition and recognition of antigen by cell-associated receptors. *Progr. Allergy* **14**. In press.