Primary Induction of Plaque Forming Antibody Producing Cells in Spleen Organ Culture* (32771)

OLE A. HOLTERMANN AND ALBERT A. NORDIN (Introduced by M. Pollard)
Lobund Laboratory, University of Notre Dame, Notre Dame, Indiana 46556

A number of reports have been published concerning induction of primary antibody responses in various tissue culture systems (1–4). The mouse spleen organ culture method employed by Globerson and Auerbach (3) has the merit of relative simplicity and permits comparatively large numbers of cultures to be set up from tissue of the same donor animal. Using sheep erythrocytes as the antigen, the above authors assessed antibody response either by direct observation of agglutination of erythrocytes in the immediate vicinity of the cultured spleen fragments, or by titration of antibody in the tissue culture fluid. Since only limited amounts of antibody can be expected to be produced by the small tissue fragments employed, it was considered likely that a more sensitive method for quantitation of the immune response could advantageously be applied to this *in vitro* system. The present report is concerned with the detection of a primary antibody response to sheep erythrocytes in mouse spleen organ culture, using the plaque technique of Jerne *et al.* (5) for enumeration of antibody producing cells. Based upon a relatively large number of observations, an assessment is given of the reproducibility of this response when measured on the fifth day after antigenic stimulation.

Material and Methods. A modification of the tissue culture system described by Globerson and Auerbach (3) was used. Briefly the system was as follows: A triangular piece of 60 mesh/inch stainless steel grid, measuring roughly 2 cm along any edge, was placed in the bottom of a 35-mm plastic petri dish (Falcon). The corners of the triangle had been bent downwards so that the central portion of the grid was supported about 2 mm above the floor of the dish. Tissue culture medium was added to the dish in a quantity (1.5 ml) sufficient to wet the undersurface of the grid without submerging it. A circular piece (1 cm diameter) of moistened Millipore membrane filter (pore size 0.45 μ) was placed on the grid. A small Teflon cylinder, 2–3 mm high and with an internal diameter of 3 mm was placed on top of the filter. A single fragment of mouse spleen tissue, measuring 1–1.5 mm on any dimension was deposited on the membrane filter in the bottom of the small culture well surrounded by the Teflon cylinder. To dishes carrying spleen fragments to be stimulated with antigen was added 25/μl of a 2% by volume suspension of sheep erythrocytes into the culture well. Nonstimulated control fragments were given the same volume of medium only. The mice were of the CFW strain, 8–10 weeks old. The culture medium consisted of equal parts of Eagle's medium (6) with nonessential amino acids added (Gibco) and medium 199 (7), supplemented with 4% agamma globulin horse serum (Hyland), sodium pyruvate 88 mg/liter and folinic acid 10 mg/liter. The medium also contained penicillin and streptomycin, 25 units and 5 μg/ml, respectively. Cultures were incubated at 37°C in a water saturated atmosphere of 95% oxygen and 5% carbon dioxide. When ready for examination, the spleen fragments were disrupted by rubbing the tissue against the steel grid and the resulting cell suspension was assayed for hemolytic plaque forming cells (5).

Results and Discussion. The kinetics of the emergence of hemolytic plaque forming cells are depicted in Fig. 1. The curves were drawn on the basis of three independent experiments. In each experiment, both stimulated and nonstimulated cultures were derived from a single spleen. Each point represents the geometric mean value of seven cultures. The general features of the curve representing the stimulated cultures are reminiscent of the kinetic curve for induction of plaque forming antibody producing cells *in vivo* (8).

* Supported by the Council for Tobacco Research, U.S.A., Grant no. 552 and by the U. S. Public Health Service, Grant no. AI07659.

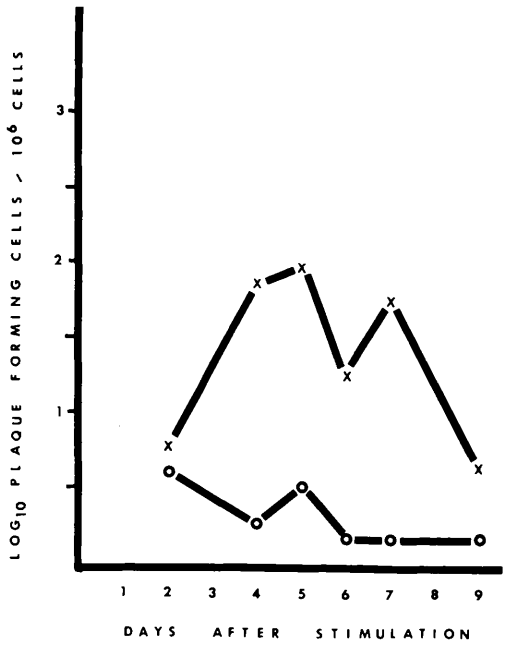

FIG. 1. The kinetics of primary induction of plaque forming antibody producing cells in mouse spleen organ cultures *in vitro*, using sheep erythrocytes as the antigen. Cultures were stimulated with antigen on day 0. Curves were drawn on the basis of three independent experiments. Each point represents the geometric mean value of 7 cultures. ×, stimulated cultures; ○, nonstimulated control cultures.

Since peak values apparently occurred on the fifth day after antigenic stimulation, it was considered of interest to investigate the reproducibility of the response on this day, using a larger number of cultures. The distribution of the plaque forming cells from 135 cultures is presented in Fig. 2. Of these cultures, 64 were nonstimulated and 71 were stimulated. Both stimulated and nonstimulated cultures were from the same 9 spleens in near equal numbers. A mean value of 0.87 plaques per 10^6 cells, with a standard deviation of 3.3[1] was calculated for nonstimulated fragments. This mean value is close to the "background" count as determined immediately upon removal of spleens from nonstimulated donors of the mouse strain used.[2]

[1] About 75% of this value for the standard deviation was due to one fragment only, showing 24 plaques per 10^6 cells.

A large spread in numbers of plaque forming cells was found among the stimulated spleen cultures ranging from < 1 to 1080 per 10^6 cells with a mean value of 112. Counts higher than 10 per 10^6 were considered significantly out of the nonstimulated range. Based on this criterion 72% of the stimulated cultures yielded counts higher than the background. This level of response was obtained without phytohemagglutinin treatment of either donor animals or cultures. Renewal of half the volume of the culture medium on the third day after stimulation did not influence the magnitude of the response as measured on the fifth day. The response was found to be specifically directed against the sheep erythrocytes used as antigen insofar that pools of cells from cultures exhibiting high counts of plaque formers towards sheep erythrocytes did not have significant numbers of plaque forming cells when tested with erythrocytes from calf, pigeon, chicken, or rabbit. Plaque forma-

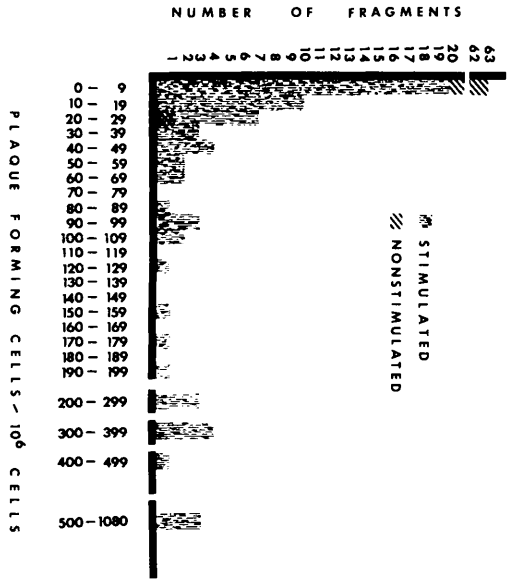

FIG. 2. Distribution of hemolytic plaque forming cells towards sheep erythrocytes among 135 mouse spleen fragments after 5 days *in vitro*. Seventy-one of the fragments were stimulated with sheep erythrocytes on the day they were established *in vitro*. The remaining 64 fragments served as nonstimulated controls.

[2] Nordin, A. A., unpublished data.

tion was dependent on the addition of active complement to the assay plate.

The vast majority of plaque forming cells recorded by the direct technique employed in the present study were undoubtedly 19S antibody producers (8). It was therefore of interest to see if application of the indirect technique would increase the number of detectable antibody producing cells (8). When applied to fragments harvested on the fifth day after stimulation with sheep erythrocytes the indirect technique did not alter the plaque counts. However, fragments which were stimulated for 9 days, showed a fivefold increase in plaque counts, indicating that at this day, in addition to 19S producers, cells producing immune globulins other than the 19S variety had appeared.

Elaboration of antibody detectable in the tissue culture fluid was tested for in a limited number of instances. Whereas agglutinating titers on the fifth day after stimulation did not exceed 2, titers as high as 64 were reached on the ninth day. This relatively high titer was obtained in cultures that had been given partial renewal of medium on the third and on the seventh day after stimulation.

The reasons for the variability of the immune response in this *in vitro* system could be manifold. One factor seems to be intrinsic in the spleen itself. Among 12 spleens tested, all yielded some fragments that did respond, however, the frequency of competent fragments ranged from 47 to 87%. Apparently the necessary apparatus for the immune response is not present in all fragments, at least when tested in the above system. Could the reason be that some of the fragments lack an essential structural unit or a clone of cells competent to respond to the particular antigen used? If such is the case, are these cells the same as the cells that register as "background" cells in the non-stimulated cultures? Contributions to the elucidation of these questions could conceivably come from a study of the relationship between the frequencies of background cells and responding fragments. In this connection germfree animals may prove to be a useful tool. Although spleens of germfree mice have a lower number of background cells to sheep erythrocytes than their conventional counterparts, the magnitude of their immune response to such erythrocytes when assayed by the hemolytic plaque technique is essentially the same.[3] It would therefore seem to be of interest to compare germfree and conventional mice with respect to frequency and topographical distribution of background cells, and immunocompetent fragments.

The primary immune response *in vitro* can undoubtedly be applied to many problems in immunology. Although the reproducibility of the response probably can be improved, use of the system described above in practical experimentation should be feasible without need for excessive numbers of cultures.

Summary. Primary stimulation with sheep erythrocytes of mouse spleen fragments *in vitro* induced plaque forming antibody producing cells. All spleens tested gave fragments that responded. The percentage of responding fragments from individual spleens varied from 47 to 87%. On the fifth day after stimulation, the numbers of plaque forming cells in stimulated cultures averaged 112 per 10^6 cells with a range from < 1 to 1080 per 10^6 cells. Values significantly above the controls were reached, on this day in 72% of the cultures. In addition to 19S antibody producers, evidence of induction of cells making other types of immune globulins was obtained. Free antibody was detected as agglutinins in the tissue culture medium.

1. Tao, T. W. and Uhr, J. W., Science **151**, 1096 (1966).

2. Bussard, A. and Hannaun, C., J. Exptl. Med. **123**, 1047 (1966).

3. Globerson, A. and Auerbach, R., Exptl. Med. **124**, 1001 (1966).

4. Mishell, R. I. and Dutton, R. W., Science, **153**, 1004 (1966).

5. Jerne, N., Nordin, A. A., and Henry, C., "Cellbound Antibodies," p. 109. Wistar Inst. Press, Philadelphia, Pennsylvania, 1963.

6. Eagel, H., Science **130**, 432 (1959).

7. Morgan, J. F., Morton, H. J., and Parker, R. C., Proc. Soc. Exptl. Biol. Med. **73**, 1 (1950)

[3] Nordin, A. A., in preparation.

8. Jerne, J., Nordin, A. A., Henry, C., Fuji, H., and Koros, A., Personal information. Information Exchange Group No. 5. Immunopathology. Scientific Memo No. 46, 1965.

Received June 7, 1967. P.S.E.B.M., 1968, Vol. 127.

Phenotypic Expression in Chickens Heterozygous for Hereditary Muscular Dystrophy* (32772)

PHILIP M. FARRELL AND LOUIS L. TUREEN

Max and Anna Tureen Neurology Research Laboratory; and the Department of Neurology and Psychiatry, St. Louis University School of Medicine, St. Louis, Missouri 63104

Hereditary muscular dystrophy in the chicken is transmitted as an autosomal recessive trait (1). Characteristic elevations in plasma creatine phosphokinase (CPK) activity resembling those in human Duchenne muscular dystrophy have previously been reported for the dystrophic chicken (2). In addition, myopathic chickens show other plasma enzymatic alterations found in the human disease, including increased activities of glutamic-oxalacetic transaminase (GOT) and aldolase (3). Recent reports have indicated that serum CPK elevations are also present in the majority of female "carriers" of human dystrophy, despite generally asymptomatic muscular systems (4,5). To determine if similar alterations were present in chickens heterozygous for muscular dystrophy, plasma CPK and GOT activities were measured in "carriers" of this myopathy.

Materials and Methods. Fifteen chickens of the New Hampshire strain were bred for the heterozygous state by fertilizing dystrophic hens with normal rooster semen (backcross technique). The birds were raised on a balanced commercial diet along with genetically normal and genetically dystrophic groups with experimental conditions as previously described (2). Between the ages of 8–20 weeks, the birds were tested serially with the "exhaustion righting test," (2,3) and scores were recorded on the basis of total successes up to ten trials. Thus a chicken which when placed on its back, rose from the supine position five times but failed on the sixth trial, was assigned a score of five. Blood samples were collected (from unexercised birds) and heparinized with the post centrifugation plasma frozen for various periods of time before assay, always well within the allotted limits (6). Serial blood specimens were taken from the heterozygotes to trace the evolution of possible CPK alterations and to achieve a representative mean over the duration of the experiment. Plasma GOT was followed serially only in the first four chicks. Heterozygote chickens were sacrificed at approximately 20 weeks of age, at which time the pectoral muscles were inspected for gross dystrophic changes (1). Specimens of pectoral muscle were taken for histological evaluation. Duplicate assays for CPK (7) and GOT (8) were performed according to the routine colorimetric procedures of the Sigma Chemical Company with sulfhydryl activation used in the CPK procedure. Revised CPK units are expressed as the phosphorylation of 1 mμM of creatine per min at 25°C being equal to 1 unit (in extrapolation, this "revised" unit is lower by a factor of 16 as compared with the previously defined unit). The GOT determinations are expressed in Sigma–Frankel units (8). All results were evaluated statistically with the paired t test.

Results. The normal chickens showed no limitation in righting themselves when placed on their backs always achieving at least a score of 10 and frequently 15–25 as previously noted by Asmundson (9). Dystrophic chicks showed impaired "righting ability," beginning at the fourth week and progressing to complete loss by the eighth to the twelfth week. The heterozygote chicks were intermediate with every chick showing a less

* This research was supported by a grant from NIH No. HD 0217.