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Studies on the Transfer of Antibody-producing Capacity

II. THE SERIAL TRANSFER OF ANTIBODY-PRODUCING CELLS*

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Summary. These studies have tested the behaviour of antibody-producing cells on serial transfer through immunologically neutral hosts. Spleen cells from rats primarily immunized *in vivo* with a *Salmonella* flagellar antigen were secondarily stimulated *in vitro* by incubation with the antigen at 37° C. They were then injected intraperitoneally into homologous rats less than 48 hours old. Two weeks later these host rats were killed, their sera titrated for antiflagellar antibody, and their spleen cells transferred to further newborn hosts, either with or without further antigenic stimulation. This type of transfer was continued for up to seven passages. It was found that if the spleen cells were not antigenically stimulated *in vitro*, they formed comparatively small quantities of antibody, and successful passage could only be achieved for two or three passages. If the spleen cells were antigenically stimulated before passage, much more antibody was produced, and successful passage could be achieved for five passages. Moreover, if the donor animal had been immunized with two unrelated antigens, and the spleen cells before passage were stimulated with one antigen but not the other, the hosts developed far more antibody to the former than to the latter.

These results are discussed in the light of recent immunological theory, and are considered to support clonal hypotheses of antibody formation.

INTRODUCTION

Antibody production and the capacity to react anamnestically persist for long periods after immunization. This persistence of specific information presents a challenging problem in somatic genetics. Recent studies in our laboratory (Nossal and Lederberg, 1958; Nossal, 1958, 1960) have shown that single plasma cells removed from animals stimulated with two or three antigens could produce only one antibody. This finding, which has been independently confirmed by other techniques (Coons, 1958; White, 1958) is consistent with recent theories on the clonal individuation of antibody-forming cells (Burnet, 1957, 1959; Lederberg, 1958, 1959; Talmage, 1957). However, such phenotypic restriction of antibody-producing capacity does not prove any genotypic restriction. This communication presents our first attempts at a study of the immunological properties of the descendants of antibody-producing cells.

In the first paper of this series (Nossal, 1959), it was shown that spleen cells from a rat primarily immunized against a *Salmonella* flagellar antigen could be secondarily stimulated *in vitro* to form antibody on transfer to homologous newborn rats. While we had no method of determining the fate of these transferred cells, it seemed reasonable to suppose that a proportion would find their way to the spleen. As such cells would not provoke immunological rejection in newborn rats (Woodruff and Simpson, 1955), they might be expected

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to survive and proliferate in much the same fashion as if they had been exposed to secondary antigenic stimulation while still in the donor animal. In the present experiments the host rats were killed 2 weeks after cell transfer, that is while they were still in the phase of immunological immaturity; their spleens were removed and splenic cells transferred to further newborn rats. Serum from each host was titrated for antibody against flagellar antigen. Passage was continued up to seven times.

These experiments bear a superficial resemblance to the experiments of Simonsen (1957), who injected chick embryos with adult fowl spleen cells or blood leucocytes, and observed the development of splenic hypertrophy and haemolytic anaemia in the hosts. However, in the present experiments interest was focused not on homograft reactions but on the persistence or otherwise of the capacity to form antiflagellar antibody. Some splenic hypertrophy was, in fact, observed in the first and second hosts but not subsequently.

MATERIALS AND METHODS

Animals

Wistar Albino rats bred by random mating and fed on a diet of mouse pellets and tap water were used. Adult rats were 3 to 5 months old at the time of injection and weighed 250–300 g. Newborn rats were always less than 48 hours and usually less than 24 hours old and weighed 5–6 g.

Antigen

Two monophasic *Salmonellae*, *S. adelaide*, flagellar antigens H_1^{fg}, and a transductional derivative of *S. paratyphi* B, SW 685, H_1^i, kindly supplied by Professor J. Lederberg, were used. Flagellar antigens were prepared as previously described (Nossal, 1959) and are referred to as 'Ad' and '685' below.

Immunization of Donor Animals

Adult rats were injected intravenously with 1.0 ml. of 1 : 400 antigens in physiological saline. Four to eight weeks later they were killed and their spleens removed aseptically.

Preparation of Spleen Cells

Spleen cells were prepared and stimulated *in vitro* as previously described, with the exception that excess antigen was not neutralized by immune serum. Cells not stimulated *in vitro* were washed once and suspended in a convenient volume of Earle's saline.

Passage of Spleen Cells

The first passage consisted of the injection of the equivalent of about 70 mg. (wet weight) of adult donor spleen intraperitoneally into each newborn rat. Rats were injected in groups of three to five. Control rats in some experiments received 0.25 ml. of 1 : 1000 antigen. Thirteen to fifteen days later, the animals were killed by exsanguination under anaesthesia, and single cell suspensions prepared from each spleen. The cells were washed once. Some suspensions were then stimulated *in vitro* and some kept at 4° C. without further treatment. All the cells from one spleen were injected into one further newborn rat, and this constituted the second passage. Thirteen to fifteen days later, this second lot of hosts was killed and the spleens treated as before. Their re-injection (one spleen per host) into further newborn rats constituted the third passage. This process was repeated for up to seven passages.

Serum Antibody Titrations

Blood samples collected from host rats during exsanguination were incubated at 37° C., centrifuged for 10 minutes, and the supernatant was titrated on the same day. The method of antibody assay, which has been described previously (Nossal, 1959) depended on the specific immobilization of *Salmonella* bacteria by antiflagellar antibody. The reciprocal of the dilution of serum giving standard immobilization was termed the titre of that serum.

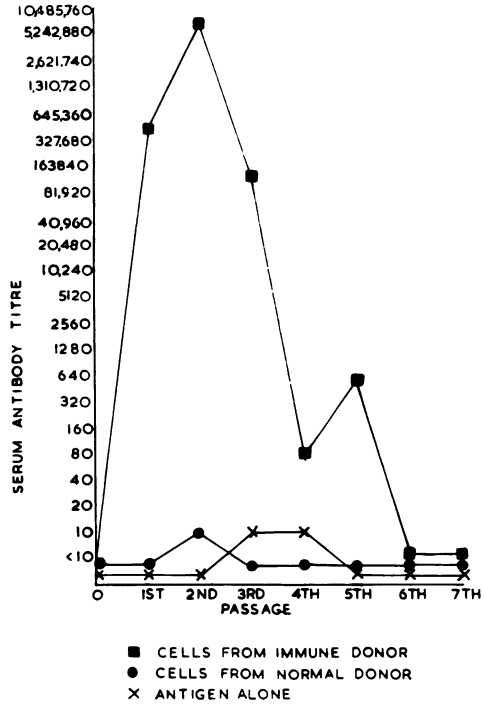

FIG. 1. Serial transfer of spleen cells stimulated *in vitro* at each passage.

EXPERIMENTAL

Preliminary experiments established that after the first injection of stimulated cells into newborn hosts, peak serum antibody titres developed after 8–12 days and were maintained for several days. No difference was observed between the titres developed by normal newborn hosts and sublethally X-irradiated newborn hosts. Thus normal hosts were used in the experiments described below.

The results of four passage series are shown in Figs. 1–4. Each point in these diagrams represents the geometric mean of the serum titres of all animals in a group. The figures along the ordinate represent successive passages. The lines join corresponding groups. In Experiment 1, the first group of newborn received cells *secondarily* stimulated *in vitro* with 'Ad' antigen, the donor having been immunized against 'Ad' 7 weeks previously. The second group received cells primarily stimulated *in vitro*, derived from a normal donor. The third group received 0.25 ml. of 1 : 1000 'Ad'. Before each subsequent passage, all the spleen cells were re-stimulated *in vitro* with 'Ad'. The experiment was continued for seven passages. The results (Fig. 1) show that neither of the latter two groups developed significant antibody, either at the first passage or subsequently. The first group developed a

mean titre of 320,000 on first passage, and of 7,000,000 on second passage. On third passage the titre fell to 120,000 and on fourth and fifth passages to considerably lower levels. On sixth and seventh passages no detectable antibody was developed.

No determinations of spleen weight were attempted during these experiments. In Experiment 1, and subsequent experiments, recipients of adult spleen cells, were they stimulated or unstimulated, developed considerable splenic hypertrophy. At the second passage, these groups developed questionable splenic hypertrophy, and at the third and subsequent passages there was no obvious splenic hypertrophy.

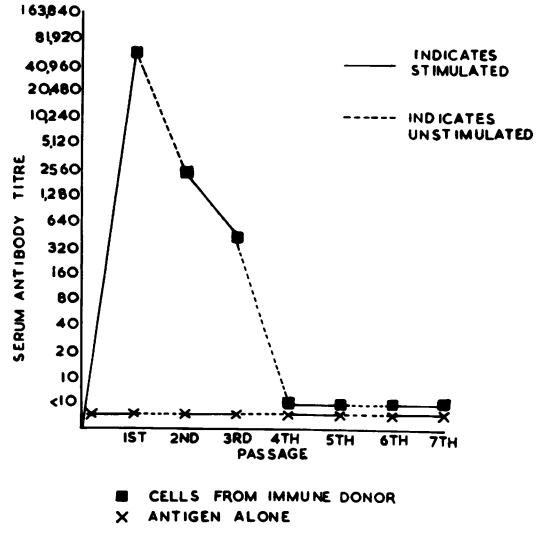

FIG. 2. Serial transfer of spleen cells stimulated *in vitro* at alternate passages.

In Experiment 2, the effect of stimulating the cells *in vitro* at alternate passages was determined. The control animals receiving 0.25 ml. of 1 : 1000 'Ad' antigen developed no antibody. The animals receiving cells secondarily stimulated *in vitro* with 'Ad' developed less antibody than in the first experiment, reaching a mean titre of 60,000 (Fig. 2). When these cells were passaged without antigenic stimulation, as indicated by the broken line, the titre reached at the second passage was only 2560. Despite antigenic stimulation before the third passage, the titre reached was only 400. Continuing stimulation at alternate passages, no antibody production could be detected in any of the next four passages.

In Experiment 3, the effect of withholding antigenic stimulation for the first two passages and then stimulating the cells at the third and subsequently at alternate passages was compared directly with the result of stimulating at each passage. It can be seen (Fig. 3) that the titres developed in hosts receiving unstimulated spleen cells from donors primarily immunized against 'Ad' were far below those developed in hosts receiving secondarily stimulated cells. While antigenic stimulation at the third passage caused a sharp rise in the antibody titre developed in the former group, the titre was still well below that of the latter group. Once again no antibody production resulted in passages after the fifth.

In Experiment 4, the donor rats were immunized with both 'Ad' and '685', and two

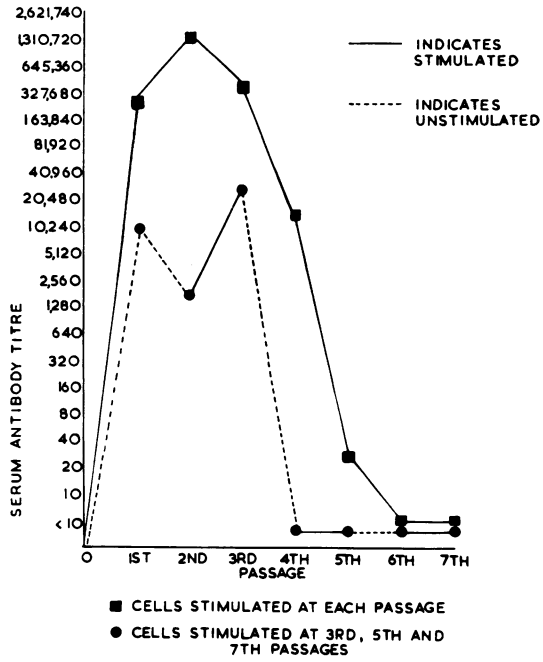

FIG. 3. Serial transfer of spleen cells stimulated *in vitro* at third, fifth and seventh passages.

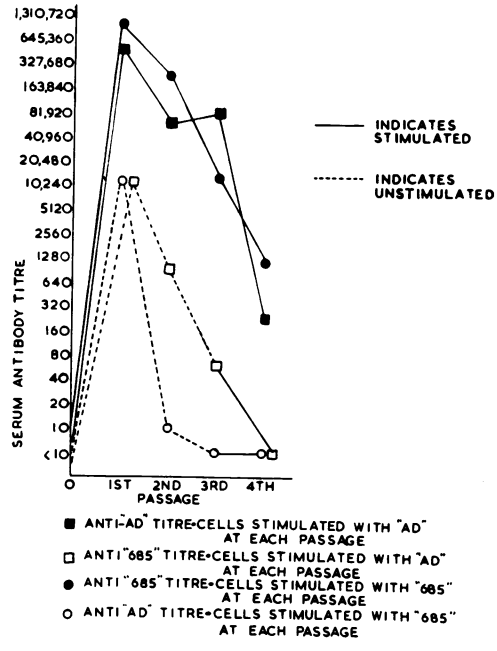

FIG. 4. Serial transfer of doubly stimulated spleen cells.

groups of hosts were injected with spleen cells. One group received cells secondarily stimulated *in vitro* with 'Ad' only; the second group received cells secondarily stimulated *in vitro* with '685' only. Before the second and third passages, cells of the first group were restimulated with 'Ad' only, and cells of the second group with '685' only. Before the fourth passage, cells in both groups were stimulated with both 'Ad' and '685'. Sera were titrated at each passage for their anti-'Ad' and anti-'685' activity. It can be seen (Fig. 4) that both groups formed more antibody in each of the first three passages to the antigen exhibited than to the antigen omitted. At the fourth passage, despite stimulation of all spleen cells with both antigens, each group developed antibody only to the antigen exhibited at each pass, and none to the antigen omitted for the first three passages.

Four other passage series have been performed to confirm the pattern of results obtained in the above four series and almost identical results have been obtained.

DISCUSSION

Our most striking finding is the persistence of the information necessary for antibody production for up to five serial passages through immunologically neutral hosts. We believe that this represents the persistence of clones of immunologically competent cells from the donor spleen with proliferation occurring in the spleens of successive newborn hosts. The spleen cells of the newborn hosts seem to play no part in our experiments. This is shown by the complete failure of control animals injected with antigen to develop antibody, either at the first passage or subsequently. Moreover, sublethally irradiated newborn animals were just as good hosts as normal newborn rats; this would be unlikely if transformation of neonatal cells by a factor present in the injected stimulated adult cells were the means of transfer of specific information. Finally, we were never able to transfer specific information by cell-free homogenates of stimulated cells (Nossal and Larkin, 1959).

In the system we have used, normal adult cells primarily stimulated *in vitro* and transferred to newborn animals do not form antibody (Nossal, 1959) nor do they lead to the development of a passageable factor in the host spleens (Experiment 1: Controls). Apparently the only cells capable of forming antibody on serial passage through newborn hosts are derived from cells specifically reactive to the antigenic stimulus given *in vivo* to the adult donor. If these cells are serially passaged without antigenic stimulation, antibody production rapidly declines and ceases after three passages (Experiments 3 and 4). Presumably the immunologically competent cells responsible have a limited natural life span and/or even more limited capacity to proliferate and undergo development to plasma cells. Moreover, their numbers will certainly be reduced at each passage because of losses of viable cells on handling and centrifugation, and because of dilution in the host. This latter factor depends on how many of the cells reach the host spleen, and how many go to other sites such as bone marrow, lymph nodes, liver, etc. However, if these cells are stimulated *in vitro* before each passage (Experiments 1 and 3) greatly increased antibody production ensues at the first passage, and on second passage even higher titres are reached. This strongly suggests that antigenic stimulation acts as a proliferative stimulus on the transferred antibody-producing cells. Apparently for the first two passages this proliferative stimulus is so strong that it overcomes the factors of damage and dilution mentioned above, so that the total antibody production rises in the second passage. However, in all experiments we observed a fall in the amount of antibody produced at the third passage, and a more pronounced fall in the fourth and fifth passages, despite repeated antigenic

stimulation. No satisfactory explanation of this phenomenon is available, but a number of possibilities can be considered, several of which are susceptible to experimental approach.

Quantitative consideration could obviously be highly significant both in regard to the size of the stimulating dose of antigen and the optimum interval between successive passages. Loss of reactivity of the cells to antigenic stimulation might be due to many factors, and it must not be forgotten that the cells responsible are being transferred to a sequence of foreign hosts. It is possible that immunological reactions between graft and host may result in damage to either or both. There may be discordance other than immunological between the transferred adult cells and the newborn hosts. The gradual exhaustion of an intracellular metabolite not produced by young animals is one speculative possibility.

We would like to stress the important role played by antigenic stimulation in our passage experiments. Omission of antigenic stimulation for only one or two passages (Experiments 2 and 3) adversely affects the subsequent response to antigenic stimulation at a further passage. This is consistent with the view that the antigen acts as a specific and essential proliferative stimulus.

Experiment 4 is of special interest. In this experiment, the donor cells were immunized against two unrelated antigens, and serially passaged three times following *in vitro* stimulation with either one or the other antigen. Those in which the spleens were stimulated with 'Ad' antigen formed large amounts of anti-'Ad', but progressively less anti-'685'; those in which the spleens were stimulated with '685' behaved in a converse fashion. Before the fourth passage, the spleens were stimulated with both antigens, but while an adequate response to the antigen exhibited at the earlier three passages was obtained, the cells failed to respond to the opposite stimulus.

These results accord well with the hypothesis that antibody-producing cells are genotypically as well as phenotypically restricted in their antibody-producing capacity. If the cells producing anti-'Ad' and their descendants were potentially capable of producing other antibodies as well on appropriate stimulation, then the failure of these cells to react to stimulation with '685' antigen at the fourth passage would be difficult to explain. But if there were two distinct cell populations, one producing anti-'Ad' and the other anti-'685', each cell handing on its specific capacity to its progeny, our results could be explained as follows: by stimulation with one antigen, the number of representatives of one population are kept high enough to form detectable antibody, even at the fourth passage. Because of failure of specific stimulation, the number of representatives of the second population is progressively reduced at each passage, so that at the end of three passages there are too few cells left to respond detectably to specific stimulation at the fourth passage.

Our results thus support the concept of the clonal individuation of antibody forming cells. However, our studies are not relevant to the important question of whether potential antibody-producing clones are present in an animal before antigenic stimulation, as in Burnet's (1959) formulation, or whether the antigen directs a specific genetic change in an undifferentiated cell. It is difficult to see any practical approach to this problem with present immunological techniques.

More recently, experiments similar to those above, but using isologous mice, have been performed. To date, these show persistence of antibody-producing capacity over eight months. The details of these experiments, which support the above conclusions, will be reported in the next paper of this series.

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