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From Cell Biology to Immunology — A Short Trip [1]

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ABSTRACT Immunologic memory and immunoglobulin allotype suppression are discussed as problems in Cell Biology. Memory, the ability of an animal after a first antigenic exposure to give a heightened and faster immune response upon a second exposure to the same antigen, is shown to be a property of bone marrow-derived cell lines. Expression of this memory depends on interaction with thymus-derived cells from either non-immunized or immunized mice.

Chronic allotype suppression is described for the first time. It is initiated by *in utero* or neonatal exposure of (SJL × BALB/c)F_1 mice, allotype a/b, to anti b antibody. Suppression lasts for long periods and continues in irradiated recipients which have received lymphoid cells from suppressed donors. Selection against b allotype producing cell precursors can explain suppression.

On arrival in 1959 in Dr. Eagle's newly formed laboratory of cell biology, it was almost immediately obvious that I had come to very fertile ground where a well-run scientific farm for research on mammalian cells had been established. Dr. Eagle had already begun to gather a superb group of young scientists with previous research experience in bacterial genetics, bacterial biology, microbial physiology, enzyme induction, microbial genetics and biochemistry and to provide an atmosphere for their talents to flower. History has shown the combination was a well chosen one.

For me, the Eagle experience proved to decisively influence the direction of my research career. The work in immunogenetics in which I have been engaged over the past several years has its roots firmly entrenched in those first crude attempts at establishing a genetics of somatic cells (Herzenberg and Roosa, '60; Roosa, Bradley, Law and Herzenberg, '62; Herzenberg, '62) begun in my term as a "two-year-wonder" at the N. I. H.

Basically, the genetics of the immune response is a subset of the genetics of somatic cells. From cell surface antigen markers on cells of a particular lineage such as the theta antigen found on cells which have passed through the thymus, to protein structural markers on immunoglobulin molecules produced by individual immunoglobulin producing (plasma) cells, the immune system provides a large variety of natural, often functional, markers for the cell geneticist to utilize. In addition, the continual process of cell differentiation which leads an animal of any age, newly exposed to a foreign antigen, not only to respond to that antigen by antibody production, but also to remember that it has seen the antigen and respond more quickly and strongly when it sees the antigen again offers excellent material for studies on expression of genetic information by somatic cells and evolution of somatic populations.

Having chosen the inbred mouse as a source of controlled genetic material, we at first concentrated on developing a formal genetics of immunoglobulin structure. Using antigenically detected protein structural markers (allotypes), we showed that there are four closely linked loci at which the structure of the constant region of the heavy chains of each of four immunoglobulin classes is defined (Herzenberg, '64; Herzenberg, Warner and Herzenberg, '65; Herzenberg and Warner, '67; Herzenberg, McDevitt and Herzenberg, '68). No polymorphism has as yet been found for γM, a fifth immunoglobulin class, nor in mice, for the light chains which appear in all immunoglobulin classes. (Parallel studies in man by Grubb, Martensson, Kunkel and

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others have shown a similar series of closely linked heavy chain loci and have shown this chromosome region to be unlinked to the genes coding for the light chain constant region.)

Work on the genetics of mouse immunoglobulin structure, which has important implications for understanding the evolution of immunoglobulins and the crucial problem of the mechanism of generation of diversity hence antibody specificity of these proteins, is continuing in our laboratory (and others, of course), but in this *festschrifte* contribution I would prefer to describe some recent studies which bear on the cellular biology of the immune response, more in keeping with the original line of interest dating from my years in direct association with Dr. Harry Eagle.

The work of Claman ('69), Taylor ('69), Miller ('69), Davies ('69) and others has shown that two type of cells are required for the production of humoral antibodies. Both of these are lineal descendents of bone marrow stem cells, but while one has no known intermediate derivation before arriving in the functional lymphoid organs of the spleen and lymph nodes, the other must pass through the thymus and is therefore called a "thymus-derived cell." Both of these cells interact with antigens, but only the former, the so-called "bone marrow-derived cell," is the precursor of antibody producing cells. What role the thymus-derived cell plays in enabling the bone marrow-derived cell to produce antibody is still unclear although it has been established that both cell lines show specificity for antigen and must be present simultaneously for a primary immune response to result.

We addressed ourselves to the question, is immunological memory, i.e., the ability to give a more rapid and heightened as well as qualitatively mature type of immune response a function of the bone marrow-derived or thymus-derived cell line or of both (Jacobson, L'age-Stehr and Herzenberg, '70). Put mechanistically, if we were to mix bone-marrow-derived cells of one type with thymus-derived cells of another, which would we have to prime in order to see a secondary response? Or, would we have to prime both?

Two prerequisites for the experimental approach we adopted were already filled in our laboratory. First, we had a pair of congenic inbred mouse strains (Klein and Herzenberg, '67; Jacobson and Herzenberg, in preparation) which were essentially isogenic (syngeneic) except for the heavy chain chromosome region. In these strains, produced by repeated backcrossing with maintenance of the marker allotype, grafts of tissues or cells are accepted without any histoincompatibility problems, although the immunoglobulins produced by the cells of each strain are distinguishable and measurable even in the presence of large quantities of immunoglobulins of the other allotype. The two strains are called CSW (immunoglobulin allotype "a") and CWB (immunoglobulin allotype "b").

Second, we had the reagents and test methods for detecting cells producing γG_{2a} antibody marked with allotype a or b (Herzenberg and Warner, '67), which not only enabled us to determine the ancestry of the cells, but also whether the cells exhibit immunologic memory, because in the protocol we use, cells producing antibody of this class (γG_{2a}) are found shortly after immune challenge only in a memory-type (secondary) response.

Our key experiment was to mix spleen cells from mice of one congenic strain which had been previously immunized (primed) with sheep red blood cells with non-primed spleen cells of mice of the second strain and inject this cell mixture along with sheep red blood cells into an irradiated recipient host of one of the congenic strains. The irradiation rendered the recipient unable to give an immune response itself, and this mouse served as an "*in vivo* test tube." The spleens of the irradiated recipients were assayed seven days later for antibody producing cells using a modification of the Jerne plaque-forming technique in which plaques are developed with anti allotype serum. Each anti allotype developed plaque is due to a γG_{2a} antibody producing cell.

We found (Jacobson, L'age-Stehr and Herzenberg, '70) that in all mixtures the γG_{2a} antibody producing cells were of the primed allotype, i.e., all the antibody producing cells were derived from the spleens of the previously immunized mice. When

CSW was primed, the plaques were a allotype. When CWB was primed, the plaques were b allotype. Although immunologically competent bone marrow-derived cells were present from the unprimed donor, they could not be recruited by the primed cells to produce γG_{2a} antibody. Since other experiments described in the same paper show that thymus-derived CSW cells can cooperate with CWB cells in both primary and secondary responses, the conclusion drawn from the above experiment is that bone marrow-derived cells must themselves be primed if they are to take part in a secondary response.

The above experiments demonstrate *only* that primed bone marrow-derived cells can make a secondary antibody response. Since thymus-derived cells were present in both the primed and unprimed spleen cell populations no conclusions can be drawn as to whether thymus-derived cells, primed or not, also are necessary for the secondary response to occur. Until a means of depleting the spleen cell populations of thymus cells was found this question remained unanswered.

In the recent collaborative experiments here and in Dr. Mishell's laboratory in Berkeley, antibody to an alloantigen, theta, which is apparently present only on thymus-derived cells (Schlesinger and Yron, '70) was used to specifically eliminate the thymus-derived cells from spleen cell populations of either immunized or non-immunized mice. (Theta bearing cells are killed *in vitro* in the presence of anti-theta antibodies and complement.) Chan, Mishell and Mitchell ('70) showed that such depleted spleen cell populations could not respond to sheep red blood cells in culture, but that addition of thymus cells restored the response. Mitchell, Chan, Mishell and Herzenberg (in preparation) showed that when the depleted spleen cell suspension was injected into irradiated recipients ("test tube" animals), the *in vivo* secondary response to sheep red blood cells was virtually completely eliminated. Adding back thymus cells taken from normal (non-primed) animals to the anti-theta treated primed spleen cell population, completely restored the secondary response (see table 1). Thus, as in a primary, thymus-derived cells are required for bone marrow-derived cells to become antibody forming cells.

To determine whether there is immunological memory in the thymus-derived cell population, it is necessary to quantitatively compare the cooperative activity of primed and unprimed thoracic duct lymphocyte populations. The experiments of this matter are continuing in this laboratory; but to-date we can say that for sheep red blood cells as the antigen, there is at most a three to fivefold increase in cooperating activity in a primed thoracic duct population as compared to an unprimed one.

To summarize, a secondary type immune response or memory response can be obtained with non-primed or primed thymus-derived cells, but only with primed bone marrow-derived cells. Unprimed bone marrow-derived cells mixed with any number of primed thymus-derived cells do not give rise to a secondary immune response. Thus, the major reservoir of immunological memory for humoral antibody is the bone marrow-derived cell population; but a cooperative thymus-derived cell population, which may increase in cooperating activity, is required for expression of this latent memory.

TABLE 1

Inhibition of memory response by depletion of thymus-derived cells using anti-theta serum and reconstitution with normal thymus cells

Treatment of immune spleen cells (a allotype)	Thymus cell supplement (b allotype)	Antibody forming cells per spleen of irradiated recipients [1]	
		a	b
Normal serum plus complement	—	5350	background
Anti theta serum plus complement	—	270	background
Anti theta serum plus complement	+	5900	background

[1] The number of antibody forming cells in recipient spleens (averages of 5) was determined seven days after lethal irradiation and injection of spleen cells plus or minus thymus cells plus antigen (sheep red blood cells).

ALLOTYPE SUPPRESSION

I wish to turn now to another area of cellular immunology in which our laboratory has been actively engaged in recent years. Our search for a system in which *in vivo* cell selection could be obtained led us to examine allotype suppression. This has been fruitful as it has proven possible to alter the normal population of immunoglobulin producing cells by the simple device of exposure to anti-allotype antibodies.

The ability to apply selective pressure to immunoglobulin producing populations arises from the unique way in which the immune system utilizes the information from alleles at a given immunoglobulin locus. Sometime early during the process of differentiation of bone marrow cells to antibody forming cells, one or the other of the two alleles at an immunoglobulin locus is excluded from functioning in the cell (Pernis, Chiappino, Kelus and Gell, '65). Thus, although allotypes are controlled by autosomal genes, in an a/b allotype heterozygote, antibody forming cells and their precursors are committed to either a or b production, never to both (for elaboration see review, Herzenberg, McDevitt and Herzenberg, '68). This yields two naturally arising populations growing concurrently in the animal, each marked by its own allotype. Production of one of the allotypes may then be suppressed at the cellular level by attack on one of the populations.

Allotype suppression was discovered in rabbits by Dray ('62). He immunized females homozygous for one allotype against the allotype of males which he then mated to these immunized females. The production of the paternal allotype was greatly suppressed in the (F_1) offspring of such matings. In some cases, this suppression lasted for months and even years.

Some years ago, my wife, working with Dr. Robert Goodlin and Edna Rivera (Herzenberg, Herzenberg, Goodlin and Rivera, '67), showed that allotype suppression can be obtained in mice as well. In the strain combination which was used, suppression was relatively shortlived. By about ten weeks of age, all mice recovered sufficiently to produce detectable amounts of circulating paternal allotypes. More recently, however, we have found that within the same allotype combination, a mothers and b fathers, changing to males of strain SJL, rather than the original C57BL/10, results in a large proportion of progeny who show suppression of paternal allotype production throughout life. Cell transfer studies with these chronically suppressed mice have provided some surprising insights into the mechanism of allotype suppression and perhaps into the normal mechanisms of control and differentiation of immunoglobulin producing cells. These interesting recent experiments have in large part been carried out by Dr. Ethel B. Jacobson (Jacobson, Herzenberg and Herzenberg, in preparation).

Figure 1 shows a comparison between the short-lived allotype suppression obtained in (C57BL/10 × BALB/c)F_1 mice and the long-term, or chronic suppression seen in (SJL × BALB/c)F_1 mice. The cumulative percentage of suppressed animals which are positive for b as a function of age are indistinguishable in the two strain combinations up to about eight weeks of age. The number of (C57BL/10 × BALB/c)F_1 mice which develop the capability of producing b then continues to increase until, eventually, all have begun b production, although their levels of b, as shown by other tests, are lower than those found in non-suppressed control mice. On the other hand, the number of (SJL × BALB/c)F_1 mice which produce Ig-1b does not increase after 8 weeks of age, and, in fact, decreases slightly with time. It is important to note that the flat portion of the curve (SJL × BALB/c)F_1 represents more or less a dynamic steady state of the population, i.e., there is permanent suppression in some mice, permanent recovery in others, but fluctuating levels in a majority of the mice.

In the extreme, chronic suppression manifests itself as the complete absence of detectable paternal allotype throughout life. Frequently though it takes a milder form in which levels of b either fluctuate or are maintained over a period of several weeks, but eventually fall permanently below detectability.

Chronically suppressed mice are not able to eliminate I^{125} labelled b globulin any faster than normal mice, precluding the possibility that these mice have somehow

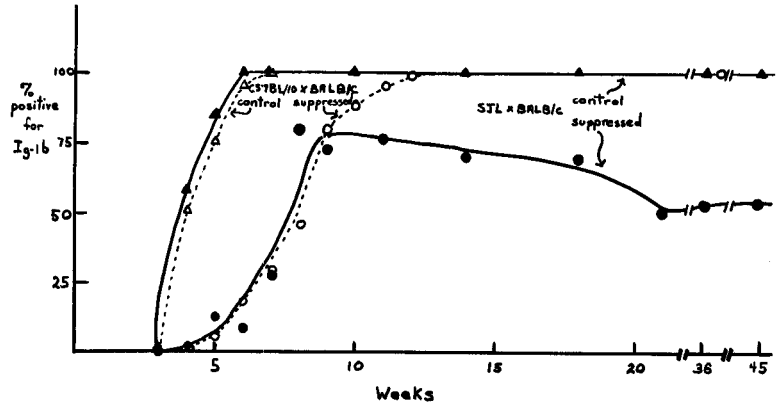

Fig. 1 Short-lived immunoglobulin allotype b suppression in one kind of a/b heterozygote compared with chronic b suppression in a second. The cumulative percentage of animals having detectable b is on the abscissa and age is on the ordinate. Triangles designate hybrid offspring of BALB/c mothers not immunized to b (controls) and circles designate hybrid offspring of BALB/c mothers actively immunized against b allotype (suppressed).

sequestered a vast store of maternal anti-b or were auto-immune to their b allotype and leaving us with the firm conclusion that these mice are permanently impaired in the ability to produce b allotype. Either most of their precursor cells committed to b are destroyed and no more can be made, or there is a continuing block which prevents b precursor cells from differentiating into b producing cells.

Since chronic suppressed animals who were negative for b allotype for weeks or even months could suddenly start producing the allotype, it seemed likely that it was not the inability to commit precursor cells to b production which was causing the suppression, but the inability to maintain such cells or to move them into allotype production. Various treatments, such as repeated bleedings or irradiation with long bone shielding, aimed at disturbing the *status quo* and forcing the animal to repopulate its lymphoid organs from early precursors were completely ineffective in restoring b production. We finally resorted to transfer of normal, syngeneic spleen cells into irradiated chronic suppressed hosts to determine whether b precursor cells could mature to b allotype producers in such an animal.

The results of this experiment are presented in table 2. Normal (non-suppressed) syngeneic spleen cells (a/b or b/b) were injected into suppressed recipients and for controls into normal BALB/c (a/a) mice. As shown in the table, the controls all had detectable circulating b allotype one week after transfer and continued to produce large amounts for the duration of the experiment. In contrast, only one of the 14 suppressed recipients had detectable b by one week. It remained positive throughout the experiment.

TABLE 2

Production of "b" allotype after transfer of spleen cells from normal (SJL × BALB/c)F_1 into suppressed and normal mice

Donor	Recipient [1]	No. positive for b allotype/total (week after irradiation)			
		1	3	5	7
Normal (SJL × BALB/c)F_1 (allotype a/b)	Suppressed (SJL × BALB/c)F_1	1/14	1/14	3/14	2/14
(SJL × BALB/c)F_1 (allotype a/b)	Normal BALB/c (allotype a/a)	8/8	8/8	8/8	8/8

[1] 600R total body irradiation was given approximately 18 hours before cell transfer to recipients.

During the course of the experiment, two more mice became positive. One of these was weakly positive at five weeks after transfer and the other had a level of b considerably below the control levels at seven weeks. The number of producers in the group is comparable with the number of chronically suppressed mice which spontaneously begin producing b allotype. Thus, exposure of a young animal to anti b allotype antibody leads in many cases to the establishment in him of a permanent suppressive environment which prevents precursor cells from differentiating to antibody forming cells which make b immunoglobulin.

Attempts to demonstrate the presence of humoral suppressing factor(s) by repeated injections of fresh serum from chronic suppressed animals into normal three to four week old heterozygotes were unsuccessful, suggesting that either cells or cell associated factors are responsible for the suppression. Transfer of spleen cells from a chronic suppressed (a/b) animal to an irradiated normal a/a (BALB/c) host, where production of b allotype by the transferred cells could be easily observed, showed that while the suppressed donor had no detectable b allotype in his own serum, he had precursor cells which produced the allotype in the free environment. However, after a few weeks, the new environment apparently became suppressing, because production of b allotype ceased, although production in recipients of unsuppressed a/b spleen or bone marrow grew stronger (see table 3). Therefore, we appear to have transferred two cell lines, one capable of producing b allotype (cryptic in suppressed animals) and the other capable of suppressing that production. The suppressing line takes some time before it prevails.

As yet we have no clear understanding of the mechanism by which exposure to maternal anti allotype antibody leads to suppression of allotype production in the young animal. The simple hypothesis that it reacts with a small amount of b allotype which appears on the surface of the precursor cell and either kills or diverts that cell so that it cannot go on to become a b producer is still attractive. If that is the case, however, we must add that, at least in the SJL hybrid and probably also in the rabbit, a second effect of the interaction between maternal antibody and b precursor cells is to stimulate the formation of a cell line which is capable, long after the maternal antibody is gone, of recognizing the b precursor and preventing it from differentiating.

TABLE 3

Production of b allotype by normal and suppressed (SJL×BALB/c)F_1

Donor cells	Spleen and bone marrow cells transferred into BALB/c [1] recipients				
	Experiment number	No. positive for b allotype/total (week after transfer)			
		2	3	5	7
Suppressed spleen	3	5/10	5/9	1/9	0/9
	4	10/10	8/9	7/9	4/9
	7	4/6	4/5	0/5	0/5
	Total	19/26	17/23	8/23	4/23
Normal spleen	3	2/2	2/2	2/2	2/2
	7	1/1	1/1	1/1	1/1
	Total	3/3	3/3	3/3	3/3
Suppressed bone marrow	3	5/6	4/5	1/5	0/5
	4	1/6	5/6	5/6	4/5
	7	4/8	3/7	4/7	4/7
	Total	10/20	12/18	10/18	8/17
Normal bone marrow	3	2/2	2/2	2/2	2/2
	7	2/2	2/2	2/2	2/2
	Total	4/4	4/4	4/4	4/4

[1] BALB/c are a/a allotype.

The reasons why SJL hybrids show chronic suppression and C57BL/10 hybrids do not may revolve around peculiarities of the SJL strain known for some time to those interested in cancer research (Murphy, '63, '69; McIntire and Law, '67; Wanebo, Gallmeier, Boyse and Old, '66). Nearly 100% of SJL males and many females over one year of age develop reticulum cell sarcomas. Younger animals frequently show severe paraproteinemias where immunoglobulins of one or another class tend to become extremely elevated, resembling a myeloma pattern on electrophoresis, or extremely depressed, resembling agammaglobulinemia, or both. Such serum patterns vary from month to month in a given animal, without apparent detriment to his health.

SJL × BALB/c animals do not develop reticulum cell sarcomas, but they do show considerably greater fluctuation in serum protein levels than do C57BL × BALB/c or other normal hybrids (unpublished observations). Whether this tendency to fluctuating serum levels makes it possible to demonstrate chronic suppression in these mice where it might be masked in other strains, or whether C57BL × BALB/c is unique in masking chronic suppression, or whether, for that matter, chronic suppression occurs in any but SJL × BALB hybrids is under study.

These short discussions of suppression and thymus-bone marrow interactions in immunologic memory are of necessity incomplete, but I hope they serve to point up some of the ways in which immunology serves cellular genetics and vice versa. It is a great pleasure to have them in a volume dedicated to Dr. Eagle because they illustrate still another field into which Dr. Eagle's influence has extended through his teaching and reasearch contributions.

Of the many people in my laboratory who have contributed to the work presented here, Drs. Ethel B. Jacobson, Graham F. Mitchell, Johanna L'age-Stehr, and of course, my wife, Lee, deserve special mention. I also wish to acknowledge the devoted help of Marion Noble, Mrynice Ravitch, Derek Hewgill, and Timothy Gadus in carrying out the experiments.

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