CYTODYNAMICS OF THE IMMUNE RESPONSE IN TWO LINES OF MICE GENETICALLY SELECTED FOR "HIGH" AND "LOW" ANTIBODY SYNTHESIS

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The basis of the immune response consists of a process of multiplication and differentiation of immunocytes induced by antigen stimulation. This process is subject to genetic control at two essentially different levels: antigen recognition and antibody synthesis. Antigen recognition constitutes the initial discriminative phase of the response which is followed by the productive phase of antibody synthesis. Genetic control of antigen recognition is generally operated by thymus-derived lymphocytes (T)[1] (1–3) while the phase of antibody synthesis depends on genetic regulation of the life cycle of bone marrow–derived lymphocytes (B) (4). The study of responsiveness to antigens of restricted heterogeneity, such as synthetic polypeptides, has led to the identification of specific immune response genes controlling antigen recognition (5).

Genetic control of the productive phase of antibody synthesis may be successfully investigated by the selection of lines of animals for the character "antibody production," in response to optimal doses of full multideterminant immunogens. The phenotypic expression of this character is represented by the peak level of serum antibody.

As the initial step of antigen recognition is no longer a limiting factor under this experimental condition, genetic regulation of the productive phase of antibody synthesis can be investigated. A "high" and a "low" responder line have been obtained from random Swiss mice by selective breeding for the amplitude of agglutinin production against heterologous erythrocytes (6, 7). The frequency distribution among the initial population of the character investigated and the progressive separation of the two lines during selective breeding indicate multiple gene control of the productive phase of antibody synthesis (6). The number of genes and loci involved will be determined by a study currently in progress on the distribution of immune responsiveness among segregating F_2 hybrids and $F_1 \times$ parental line backcrosses.

The group of genes segregated in each line by selective breeding regulates the amount of antibody produced in response to many unrelated antigens such as: sheep and pigeon erythrocytes, somatic (O) and flagellar (H) antigens of Salmonella typhi (6), hen ovalbumin (8), Limulus polyphemus hemocyanin, bovine serum albumin (4),

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[1] Abbreviations used in this paper: B lymphocytes, bone marrow–derived lymphocytes; BSA, bovine serum albumin; PFC, plaque-forming cells; RFC, rosette-forming cells; SE, sheep erythrocytes; T lymphocytes, thymus-derived lymphocytes.

dinitrophenyl hapten,[2] pneumococcal polysaccharide[3] and T_4 bacteriophage.[4] The two lines of mice also differ in their ability to synthesize humoral antibody against histo-compatibility antigens (4), antibody enhancing tumor growth,[5] and in the incidence of benzopyrene-induced tumors (9). The difference in immunoresponsiveness of the two lines involves the production of various classes of antibody: 19S, 7S γ_2, 7S γ_1, and reagins (6, 8). A marked interline difference in the serum concentration of all classes of immunoglobulins has been demonstrated after antigen stimulation; the levels are uniformly lower in the low responder than in the high responder mice (7). All these data suggest that selective breeding for responsiveness to multideterminant immunogens has resulted in the segregation of genes concerned with the general regulation of immunoglobulin synthesis irrespective of immunological specificity.

The phenotypic expression of these genes may be easily and quantitatively assessed by studying the production of agglutinins against sheep erythrocyte which has been the principal immunogen used during selective breeding (6). A reasonable hypothesis to explain all the above mentioned findings is that the group of genes segregated in each line would regulate the rate of multiplication and differentiation of antibody-producing cells after antigen stimulation. Experiments were therefore undertaken to study the dynamics of the immune response in high and low responder mice at both cellular and humoral levels. The rate of differentiation of the cells involved was investigated by studying the morphology of rosette-forming cells at the end of the exponential phase of the immune response. The results reported in this paper are compatible with the above mentioned hypothesis.

Materials and Methods

Animals.—Mice used belonged to high and low responder lines separated by selective breeding for the character agglutinin production to heterologous erythrocytes as previously described (6, 7). Adult animals of both sexes from the 16th, 18th, and 20th generations of selection (F_{16}, F_{18}, F_{20}) were used. When not otherwise stated, each experiment was performed with groups of 10–15 mice.

Immunization.—*Antigen*: Washed fresh sheep erythrocytes (SE) were injected at the dose and by the route indicated in each experiment.

Adjuvants: Difco complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) and a saline suspension of heat-killed *Corynebacterium parvum* (*C. parvum*) were used as indicated.

Antibody assay: Pooled sera from each experimental group were used. The antibody titer was expressed either as the highest serum dilution giving a positive result or as the $\log_2$ of this dilution starting from 1 = 1/10 dilution.

Agglutinin titers were determined by a microagglutination technique in standard plates: 0.05 ml of SE suspension (2×10^8 cells/ml) in buffered saline (pH 7.3) was added to 0.05 ml of doubling serum dilutions. Agglutination was read 24 hr afterwards.

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[2] Del Guercio, P., and H. Zola. Manuscript submitted.

[3] Howard, J. G., G. H. Christie, B. M. Courtenay, and G. Biozzi. Manuscript submitted.

[4] Howard, J. G. Personal communication.

[5] Biozzi, G., C. Stiffel, D. Mouton, Y. Bouthillier, and C. Decreusefond. Manuscript submitted.

Hemolysin titrations were carried out in test tubes; 0.1 ml of doubling serum dilutions in saline was mixed with 0.1 ml of SE suspension containing 1×10^9 SE/ml, then 0.6 ml of a 1/50 saline dilution of fresh guinea pig serum was added. The tubes were incubated at 37°C for 30 min. After centrifugation the serum dilution giving 50% hemolysis was determined.

Enumeration of Spleen Cells Involved in the Immune Response to SE.—Two methods have been used: the hemolytic plaque technique for detecting the plaque-forming cells (PFC) and immunocytoadherence for detecting rosette-forming cells (RFC). Isolation and quantitation of spleen cells were carried out as previously described (10). The numbers of PFC and RFC were established from the means of duplicate samples.

Enumeration of PFC: Direct PFC were detected by a modification of the Jerne method (11). Medium 199 was used throughout. Suitable numbers of spleen cells were mixed with a suspension of SE in 1% agarose kept at 45°C in a water bath. The suspension was immediately spread on a warm microscope slide to obtain a 2 mm thick preparation. After incubation for 1 hr at 37°C in a humid atmosphere (4% CO_2 in air), the slides were covered with a 10% dilution of fresh guinea pig serum (absorbed with SE and kept frozen at -70°C). Slides were then incubated at 37°C for a further 30 min before PFC enumeration under slight magnification.

Enumeration of RFC: RFC were detected by the method previously described (10, 12) by which less than 3% of RFC are attributable to cells passively sensitized by cytophilic antibody (13, 14). Higher percentages observed by other investigators are due to the use of methods which are less inhibitory to the participation of macrophages in rosette formation (15–17).

Morphology of RFC: Cell suspensions containing RFC were spread on slides and stained by the May–Grunwald–Giemsa technique as previously described (14). The percentage of each type of RFC was established after checking at least 80–100 RFC. The very few rosette-forming macrophages were excluded.

RESULTS

Separation of High and Low Responder Lines during Selective Breeding.— The mice used in the present study resulted from a process of genetic selection for the character agglutinin production against heterologous erythrocytes continued for 20 consecutive generations.

Fig. 1 A shows the resultant separation of high and low responder lines during selective breeding for 20 generations in terms of the peak agglutinin levels, induced by an optimal dose of SE. As previously described (4, 6) the antigen used for the first six generations was SE, leaving a 30 day interval between weaning and immunization. As under these conditions passively transmitted maternal antibody interfered with the immune response of the progeny in the high line, these initial results are represented by a broken line in Fig. 1 A. After it was found that the selection operated also for the response to antigenically unrelated pigeon erythrocytes, the two antigens were alternated from the 6th generation onward to eliminate the specific interference of maternal antibody (7). For simplicity only the responses to SE are represented. The extent of interline separation can be correctly appreciated from the 6th generation onward when the interfering effect of maternal antibody on the response of high responder mice was eliminated.

The separation of the two lines has been progressive: the intensity of the response decreased in the low line until the 14th generation and thereafter re-

mained at a minimal level, whereas the gain in responsiveness in the high line persisted until the 18th generation.

Individual variations in the starting population were large and continuous as shown in the frequency distribution curve (Fig. 1 B). This fact, in addition to the progressive interline separation produced by selective breeding, indicates clearly that the character investigated is determined by the cumulative effect of several quantitative genes. Around the 16th–18th generations, the interline
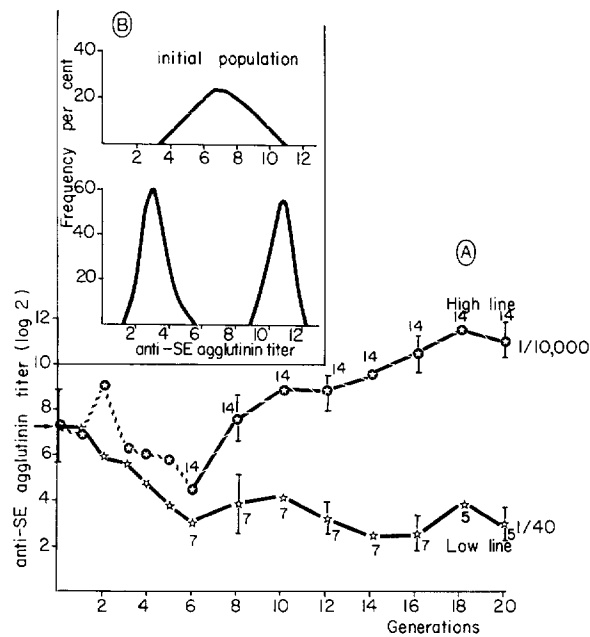

Fig. 1. (A) Separation of high and low responder lines of mice during the selective breeding. The day of the maximal agglutinin titer is indicated at each generation. The vertical bars indicate standard deviation. (B) Frequency distribution of agglutinin titers in mice of the initial population and in F_{20} mice of the high and low responder lines.

separation seems to have reached its maximal extent. The range of individual variability decreased progressively throughout breeding as progressive homozygosity was established in the two lines with regard to the character agglutinin production. The frequency distribution curve of F_{20} mice (Fig. 1 B) shows a high degree of homogeneity. These data imply that all the genes determining the character investigated have segregated in each line by the 20th generation, so that the mice can therefore be considered as homozygous in this respect.

Agglutinin Production by High and Low Responder Mice after Different Doses of Sheep Erythrocytes.—To undertake the dynamic study of the immune response it is important to determine the intensity of the responses in relation to

the dose of antigen administered (18). This study of dose-response relationship is represented in Fig. 2. The dose of 10^4 SE produced no detectable increase in the titer of natural agglutinins ($<1/10$) in both lines and can therefore be considered as subimmunogenic, whereas 10^5 SE stimulated a detectable agglutinin synthesis only in the high line.
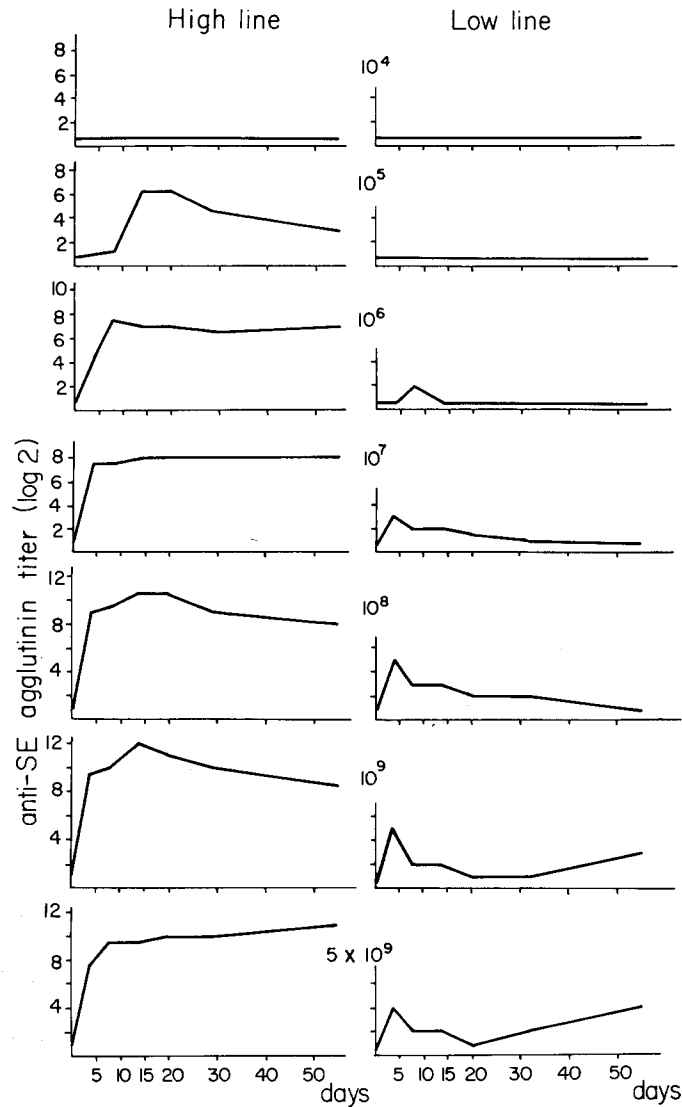

FIG. 2.   Dynamics of agglutinin production after intravenous immunization with increasing doses of SE in F_{16} mice of high and low responder lines.

Larger doses of antigen (10^6) are immunogenic in both lines, although the immune response is far stronger in the high than in the low line. The threshold of antigen stimulation is therefore an order of magnitude lower in the high than in the low line. High responder mice show prolonged agglutinin production reaching peak levels between 8 and 56 days postimmunization while low responders show a more transient response characterized by an early peak on the 4th day. For both lines, the intensity of the immune response, as measured by
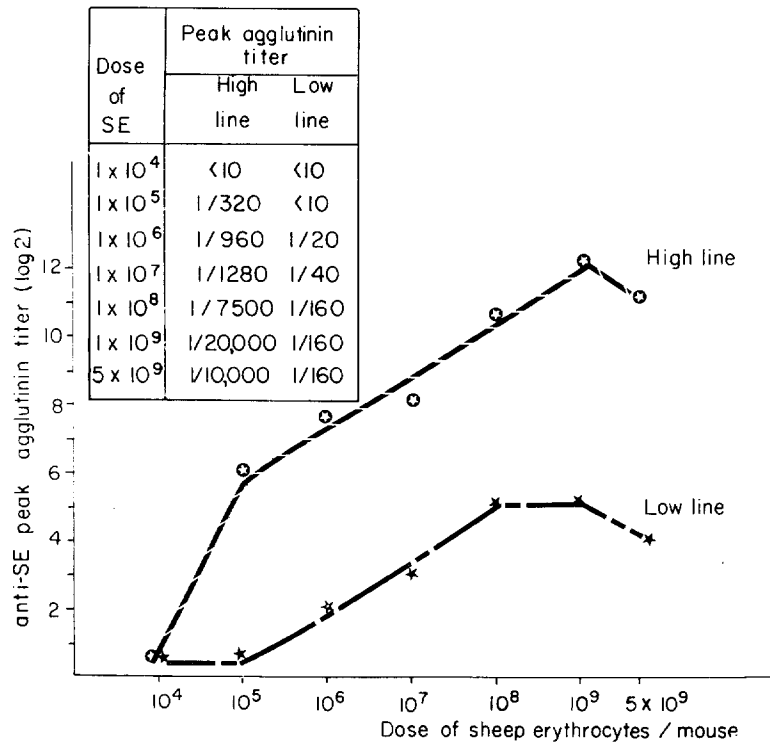
Dose of SE	Peak agglutinin titer	
	High line	Low line
1×10^4	<10	<10
1×10^5	1/320	<10
1×10^6	1/960	1/20
1×10^7	1/1280	1/40
1×10^8	1/7500	1/160
1×10^9	1/20,000	1/160
5×10^9	1/10,000	1/160

Fig. 3.   Relationship between the immunizing dose of SE and the peak titer of anti-SE agglutinins produced in F_{16} mice of high and low responder lines.

the peak level of agglutinin, is directly proportional to the dose of antigen until an optimal dose of 10^9 SE for the high line and of 10^8 SE for the low line. The dose-response relationships in both lines are summarized by plotting the peak agglutinin titers against the corresponding doses of antigen (Fig. 3).

Over the range of the dose-response relationship the magnitude of the response is a direct function of the amount of antigen injected, a function which is of the same order in both lines since the two curves have the same slope. A noteworthy interline difference is the greater sensitivity to antigen stimulation of high responder mice which also require a larger dose of antigen to give the

maximal response, indicating a broader dose-response relationship. It should be mentioned that 5×10^9 SE is the maximal dose that can safely be injected intravenously into the mouse.

The interline difference in the peak agglutinin levels induced by optimal stimulation is about 125-fold. Nevertheless, it would be incorrect to assume that this represents a real measure of the maximal separation in immune responsiveness between the two lines. In fact, it has been demonstrated that the antigen stimulation produced by SE injected intravenously in the mouse is of short duration. The ascending phase of a response at the cellular level stops at the 5th day postimmunization for lack of continuing antigen stimulation. If a supply of
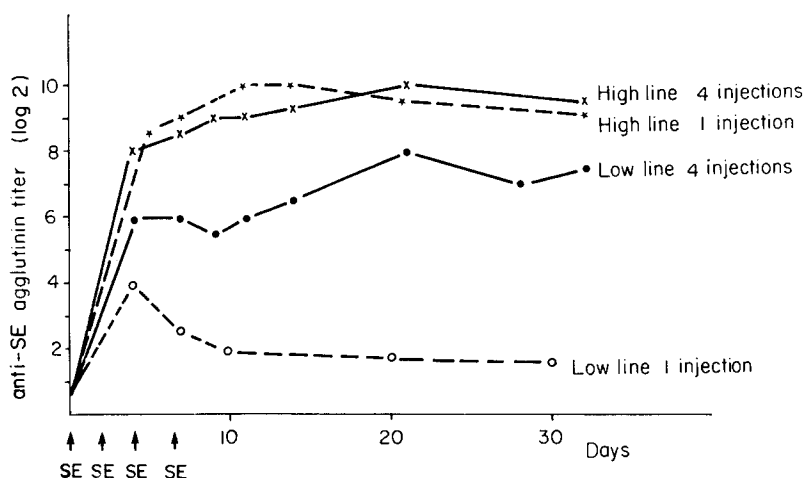

FIG. 4. Dynamics of agglutinin production in F_{18} mice of high and low responder lines after one or four intravenous injections of 5×10^8 SE. The arrows indicate the times of SE injection.

antigen is given, the response can be prolonged until maximal responsiveness is attained (12). To appreciate the full responsiveness of high and low responder mice, agglutining production was studied after repeated antigen injections and compared with the effect of a single dose of antigen (Fig. 4).

In the high line, agglutinin titers were similar in animals immunized intravenously with one or several injections. In contrast, repeated injections of antigen induced a higher and more prolonged response in the low line. The peak interline difference of about 125-fold after a single immunization is reduced to only eightfold after repeated injections. The duration of the rapid exponential rise of agglutinins lasts for about 4 days in mice of both lines receiving one or several antigen injections. After this phase, the rise in serum agglutinin continues at a slower rate for 20 days in low responders receiving repeated injections. These findings indicate that the effect of antigen stimulation is of shorter duration in low responder mice.

Effect of Adjuvants on the Responsiveness of High and Low Responder Mice to Sheep Erythrocytes.—It is well established that the immune response to particulate antigens injected intravenously is localized principally in the spleen (19, 20). Previous experiments have demonstrated that *Corynebacterium parvum* is a potent adjuvant of the immune response (21). A saline suspension of *C. parvum* injected intravenously in the mouse produces marked splenomegaly accompanied by a strong increase in the magnitude of responsiveness to SE at both humoral and cellular levels (agglutinin and rosette-forming cells) (22).
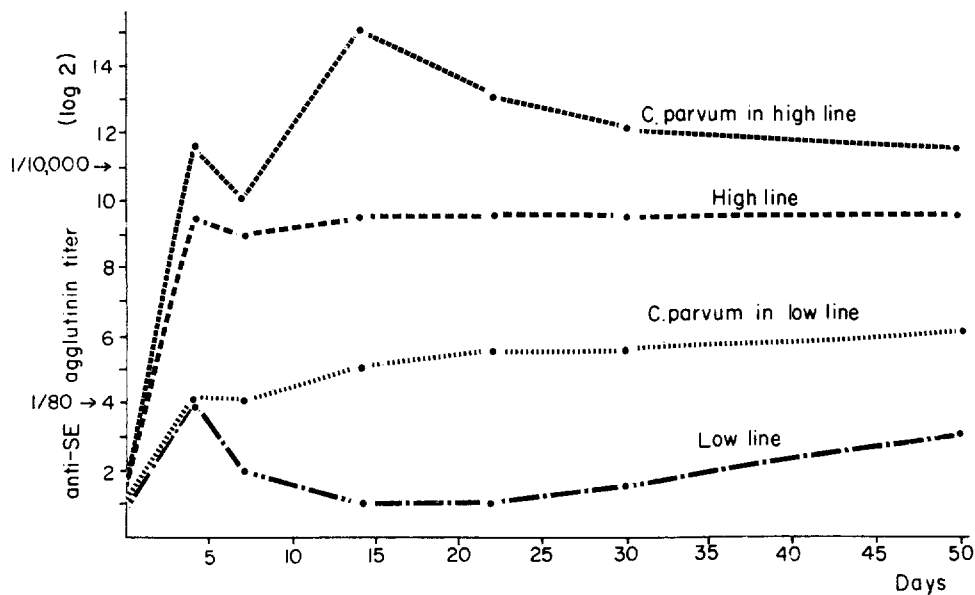

Fig. 5. Effect of *C. parvum* treatment on anti-SE agglutinin production in F_{16} mice of high and low responder lines. 0.5 mg of *C. parvum* was injected intravenously 4 days before 5×10^8 SE.

Cytodynamic analysis revealed that this adjuvant effect is due to prolongation of the exponential increase in the antibody-forming cell population in the spleen (12).

Fig. 5 shows the adjuvant effect of *C. parvum* on agglutinin production against SE in high and low responder mice of the 16th generation. Two groups of five mice of each line were immunized intravenously with 5×10^8 SE and compared with two other equivalent groups which received 0.5 mg of *C. parvum* intravenously 4 days before the same dose of SE. The effect of the adjuvant was very reduced in the low line, where it only prolonged the persistence of the maximal agglutinin level. On the contrary, *C. parvum* increased markedly the peak level attained in the high line. The interline difference in animals treated by the adjuvant reached 1000-fold as compared with the normal 125-fold.

The difference in responsiveness between the high and the low lines is also observed when the immunization is performed by a subcutaneous route as shown in Fig. 6. In this experiment SE in complete Freund's adjuvant were injected into the two hind footpads, the local persistence of antigen producing
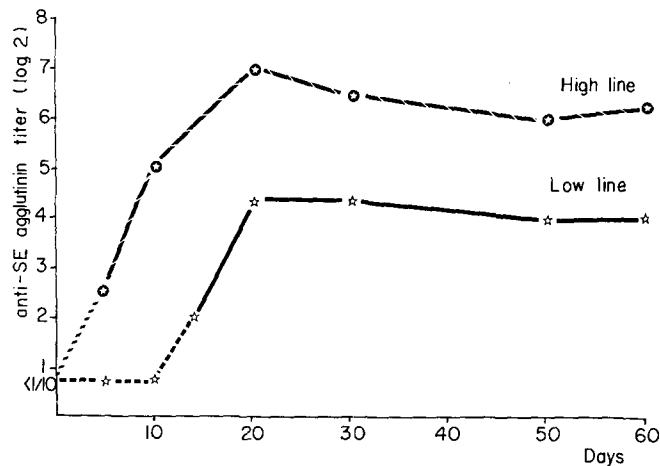

FIG. 6.   Dynamics of anti-SE agglutinin production in F_{16} mice of high and low responder lines after subcutaneous immunization in the two hind footpads with SE emulsified in complete Freund's adjuvant. 5×10^7 SE in 0.05 ml of emulsion were injected into each footpad.
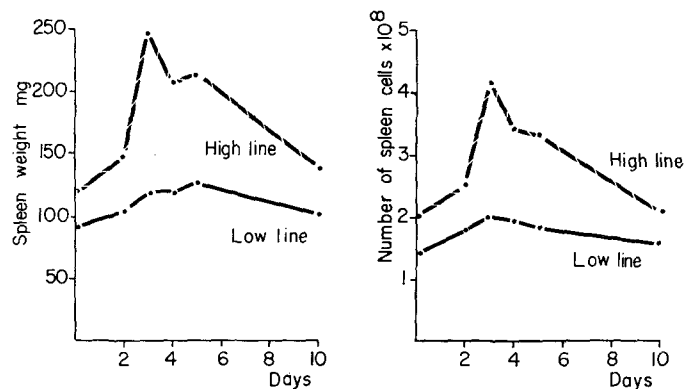

FIG. 7.   Spleen weight (left) and total number of isolated spleen cells (right) in F_{16} mice of high and low responder lines immunized with an intravenous injection of 5×10^8 SE.

a prolonged stimulation of the regional lymph nodes. Initially, the agglutinin titers were very different in the two lines. A substantial amount of antibody was already synthesized by day 5 in the high line, while no significant response was detected in the low line until day 14. Subsequently, a rise in agglutinins occurred also in the low line mice, which eventually produced a substantial

amount of antibody as compared with high responders. Consequently, the interline difference at the peak levels was smaller than that observed after intravenous immunization (see Figs. 2 and 3).

Kinetics of the Immune Response in High and Low Responder Mice.—F_{16} mice were immunized intravenously with the optimal dose of 5×10^8 SE and the number of spleen cells induced into immune response was established by measuring the numbers of rosette-forming cells (RFC) and plaque-forming
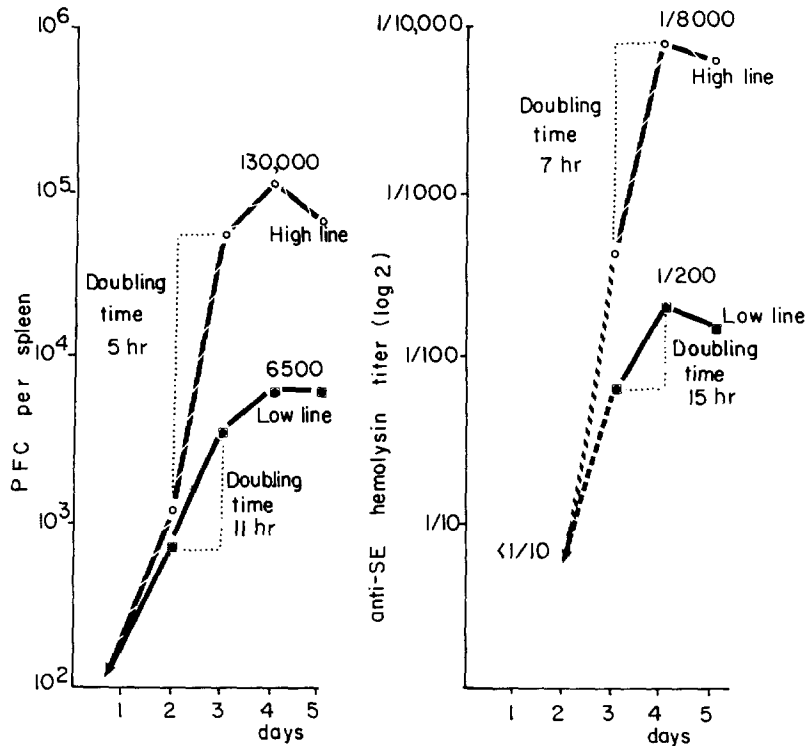

Fig. 8. Dynamics of the response measured in terms of PFC (left) and of anti-SE hemo lysin (right) in F_{16} mice of high and low responder lines immunized with an intravenous injec tion of 5×10^8 SE.

cells (PFC). RFC represent the totality of the spleen cell population engaged in all aspects of the immune response, whereas PFC comprise those cells engaged in the secretion of a large amount of hemolytic antibody (22–30). Groups of 10 mice of each line were killed at different times after immunization and the spleen weight and the yield of isolated spleen cells were determined (Fig. 7). The isolated cells were distributed in two aliquots, one used for PFC assay (Fig. 8) and the other for RFC assay (Fig. 9). Agglutinin and hemolysin titrations were made on the pooled sera of each group. In the present study only

the exponential phase of immune response (lasting 4–5 days postimmunization) is considered. At the end of this phase the interline difference is almost fully expressed at both humoral and cellular levels. The mean values of spleen weight and cellularity before and after immunization are represented in Fig. 7.

Spleen weights before immunization are somewhat smaller in the low line than in the high line. Antigen stimulation produced a marked increase in the spleen weight in the high line where the spleen cell population doubles in 3 days. Such an effect was much smaller in the low line where the spleen size increased
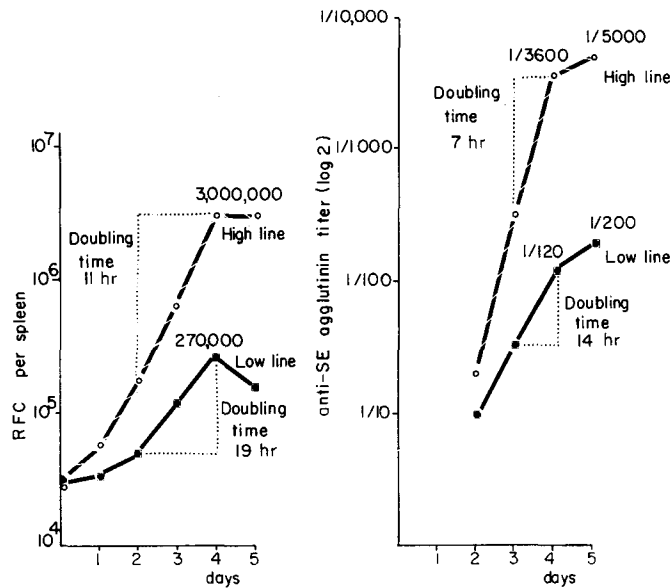

FIG. 9. Dynamics of the response measured in terms of RFC (left) and of anti-SE agglutinin (right) in F_{16} mice of high and low responder lines immunized with an intravenous injection of 5×10^8 SE.

by only about 40% in terms of both weight and cell number. The immune response measured in both lines by the mean number of PFC per spleen and the mean titer of serum hemolysin is represented in Fig. 8.

The number of PFC and the hemolysin level before immunization were similar in both lines. The increase in PFC number and rise of serum hemolysin occurred faster and reached higher levels after immunization in the high than in the low line. The rate of the response during the exponential rise can be measured for PFC number and hemolysin titers by the doubling time. Comparison of both of these doubling times indicates that the rate of the response in the high is about twice that in the low line (see Table I and Fig. 8).

The interline difference at the end of the exponential phase is greater at the

humoral than at the cellular level. This difference is about 40-fold for hemolysins and 20-fold for hemolysin-secreting cells (PFC). Since both methods are based on direct hemolysin reaction and there is no extra splenic localization of PFC at this period, the figures obtained can be compared directly. Such a comparison indicates that the mean amount of hemolysin produced by each PFC is about twofold larger in the high than in the low responder mice. Thus the genetic constitution of each line regulates both the number of PFC and the amount of hemolysin secreted per cell after antigenic stimulation. The immune responses measured in both lines by RFC per spleen and by agglutinin titers are shown in Fig. 9.

TABLE I

Comparison of Relevant Immunological Parameters in Mice of High and Low Responder Lines

		High line	Low line	High line/ Low line
Nonimmunized mice	Natural PFC/spleen	$\leqslant 70$	$\leqslant 70$	
	Natural RFC/spleen	23,000	30,000	
	Natural serum hemolysins	<1/10	<1/10	
	Natural serum agglutinins	1/10	1/5	
4th day postim-	PFC/spleen	130,000	6,500	20
munization	RFC/spleen	3,000,000	270,000	11
5×10^8 SE i.v.	Serum hemolysins	1/8000	1/200	40
	Serum agglutinins	1/3600	1/120	30
Doubling time of PFC		5 hr	11 hr	0.45
Doubling time of RFC		11 hr	19 hr	0.58
Doubling time of serum hemolysins		7 hr	15 hr	0.47
Doubling time of serum agglutinins		7 hr	14 hr	0.50

The RFC levels and the agglutinin titers found before immunization were similar in both lines. The rate and magnitude of the response after stimulation are markedly greater in the high than in the low line. As in the preceding experiment (Fig. 8) the interline difference, at the peak of the response is greater at the humoral than the cellular level. The principal parameters resulting from the experiments represented in Figs. 8 and 9 are summarized in Table I.

No significant difference was observed in the titers of natural antibodies and in the levels of PFC or RFC found in the two lines before immunization. Although the rise in the number of PFC or RFC in the spleen is not a simple exponential function during the ascending phase of the immune response, the maximal rate can be estimated from the doubling times on the steepest part of the curves represented in Figs. 8 and 9. The values of the ratio between the rates of immune responses in low and high lines measured in terms of doubling times of PFC, RFC, hemolysins, or agglutinins are very similar and close to 0.5

(0.45–0.58). This means that the speed of the immune response is twofold higher in the high than in the low responder mice. This kinetic factor accounts for the interline difference observed at the peak of the cellular response on the 4th day. Comparison of the immune responses at this time shows that the interline difference is greater at the humoral (30–40-fold) than at the cellular (10–20-fold) level.

In the same spleen cell population, the number of RFC is always larger than that of PFC, particularly in nonimmunized mice where the background of
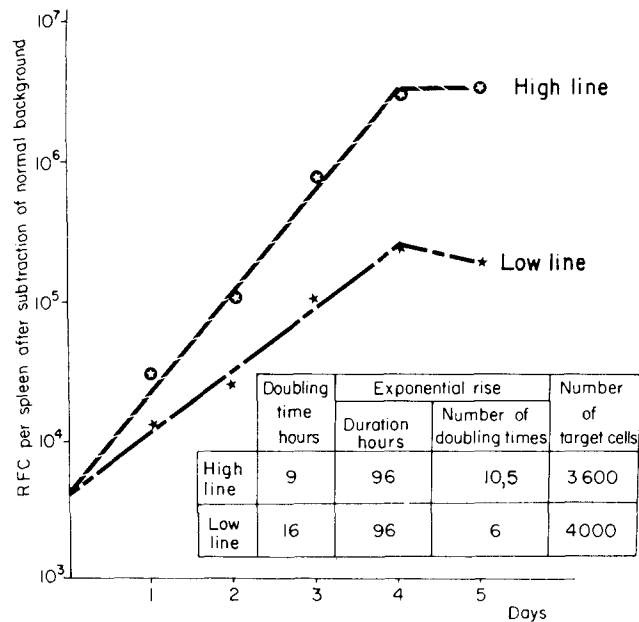

Doubling	Exponential rise		Number
time hours	Duration hours	Number of doubling times	of target cells
High line	9	96	10,5	3600
Low line	16	96	6	4000

FIG. 10. Cytodynamic constants established in F_{16} mice of high and low responder lines immunized with an intravenous injection of 5×10^8 SE. The curves are constructed by subtracting the background of natural RFC (Table I) from the experimental data presented in Fig. 9.

natural RFC is more than 400-fold higher than that of PFC (12, 13). This large background of RFC interferes with accurate determination of the initial phase of the immune response (12) as the number of RFC produced by antigen stimulation at the onset of the response is small. This interference can be eliminated by subtracting the natural background from the experimental points of the ascending phase of the response. After correction, the rise in RFC in the spleen follows a strict exponential function indicating a constant rate of expansion in the RFC population which provides an accurate measurement of the RFC doubling time. In addition the number of "target" cells initially stimulated by the antigen can be calculated by extrapolation on the ordinate to zero

time (12). When immunization is performed with an optimal dose of antigen, the number of target cells represents the number of spleen cells able to respond to this antigen at the time of immunization (13, 18).

Fig. 10 shows the ascending phase of the response in high and low lines corrected for normal RFC background. The basic difference between the two lines concerns the doubling time of RFC which is about twofold shorter in the high than in the low line (4).

Other cytodynamic parameters, such as the number of target cells and the duration of the exponential phase, are similar in both lines. Therefore, the interline difference in the number of RFC found at the peak of the response is attributable to a single factor: the RFC doubling time. The ascending phase of the response in the high line is constituted by an initial population of 3600 cells which, on stimulation by antigen, double every 9 hr for 10–11 times consecutively to reach the peak of the response. In the low line a similar number of cells (4000) are initially stimulated by the antigen but their expansion stops after six consecutive doubling periods of 16 hr each. The value for doubling time of RFC presented in Fig. 10 is in agreement with the evaluation of the same parameter made on the steepest part of uncorrected curves reported in Fig. 9.

Morphological Study of RFC in the High and Low Responder Lines.—The following morphological types of RFC are identifiable in stained preparations by light microscope: small, medium-sized, and large lymphocytes, blast cells, and plasma cells (14). This classification has been confirmed by electron microscopy (14). The distribution of the different types of RFC in nonimmunized mice and on the 4th day postimmunization was established on aliquots of the same spleen cells used for the cytodynamic study represented in Fig. 9. The results obtained are summarized in Table II. About 90% of natural rosettes found in nonimmunized mice are small lymphocytes, while the remainder are medium-sized lymphocytes of rather small size. No significant interline difference was observed in the morphology of natural RFC. By contrast, significant differences between the two lines were observed at the end of the exponential rise of RFC (4 days postimmunization). There was a marked increase in the percentage of plasma RFC in the high line. The compensatory decrease in the other types was particularly evident for small lymphocytes. The increase in the percentage of plasma cells in the high line was about fourfold but allowing for the absolute number of RFC and the size of the spleen cell population, the total increase in plasma cells is 43-fold in favor of high responder mice. On the contrary, other cell types increased only to about the same extent as the total number of RFC (11-fold). This demonstrates a preferential increase in plasma cells among RFC in the high line.

The interline difference in the level of serum antibody (30–40–fold) is of the same order as that of plasma cells (43-fold), suggesting that the former are secreted by the RFC classified as plasma cells while antibody synthesized by

other cell types is apparently not secreted. Comparison of the data reported in Tables I and II shows that the number of plasma RFC greatly exceeds that of PFC, so that not all the former secrete enough antibody to produce a hemolysis plaque under our experimental conditions. (A considerably larger number of PFC can be detected by improving the sensitivity of the method [29].) These

TABLE II

Morphological Study of RFC in Mice of High and Low Responder Lines before Immunization and 4 days after i.v. Immunization with 5 × 10⁸ SE

		High line	Low line	High line/ Low line
Immunized mice	No. of spleen cells	344×10^6	195×10^6	1.8
	RFC/1000 spleen cells	8.7	1.4	6.2
	Total rosettes per spleen	3×10^6	270×10^3	11
	Serum hemolysins titer	1/8000	1/200	40
	Serum agglutinins titer	1/3600	1/120	30

	Morphology of RFC	%	Total per spleen	%	Total per spleen	Total high line/ Total low line
Immunized mice*	Small lymphocytes	10	300,000	20	54,000	5.5
	Medium-sized lymphocytes	28	840,000	26	70,500	12
	Large lymphocytes	16	480,000	22	59,500	8
	Blast cells	22	660,000	26	70,500	9
	Plasma cells	24	720,000	6	16,500	43
Nonimmunized mice‡	Small lymphocytes	87	19,900	88	26,200	
	Medium-sized lymphocytes	13	3,100	12	3,800	

* Number of rosettes examined: 1070 in high line and 560 in low line.

‡ Number of rosettes examined: 80 in each line.

data explain the discrepancy in the interline difference between RFC and serum antibody found in the experiments represented in Fig. 9.

It is highly probable that the different types of RFC found during the immune response derive from small lymphocytes preexisting in nonimmunized mice through a process of morphological differentiation cumulating in the plasma cells. This process of cell differentiation occurs at a faster rate in high responder mice (Table II). The percentages of medium-sized lymphocytes, large lymphocytes, and blast cells are similar in the two lines; therefore, the acceleration of the process of cell differentiation which characterizes the high responder mice

appears to involve essentially the maturation of specific immunocytes into plasma cells. The morphological observations are compatible with the hypothesis that the group of genes segregating in each line regulates the rate of differentiation of antibody-producing cells after antigen stimulation.

DISCUSSION

The initial population of random-bred mice used for selective breeding presented large and continuous variations in antibody titers of individual animals (Fig. 1 B). The frequency distribution curve representing this variation suggests polyfactorial regulation of the productive phase of the immune response. The genetic control of these factors is demonstrated by the progressive separation of high and low responder lines during selective breeding. The difference in responsiveness between high and low lines cannot be explained in terms of progressive accumulation or loss, respectively, of specific immune response genes each controlling the response to a single determinant present in the complex antigenic mosaic of SE, as the separation extended to other immunologically unrelated immunogens.

F_{16}, F_{18}, and F_{20} mice used in the present study showed a remarkable interline separation and small intraline variability of their immune responsiveness to SE (Fig. 1 B). The difference in the amount of antibody produced by the two lines was verified for a full range of antigen doses, from minimal to maximal. No immune tolerance could be produced by the largest doses of SE. Only a plateau zone of antigen excess was observed in both lines, where responsiveness is no longer a function of antigen dose (Fig. 3).

Sobey et al. (31) separated by genetic selection a line of low responder mice to bovine serum albumin (BSA). This antigen administered in large amounts produces specific tolerance and the low responsiveness of Sobey's mice was, in fact, determined by a lowered tolerance threshold since they respond normally to lower doses of BSA (32). The difference in the amounts of agglutinins synthesized by our high and low responders was the same over the entire zone of dose-response relationship, so that the difference is in their ability to synthesize antibody rather than in their susceptibility to tolerance induction. Nevertheless, a smaller amount of antigen is required by the low responders to reach an optimal response. These mice also show a decreased susceptibility to antigen stimulation as shown by the higher threshold dose of antigen. A similar difference has been found by Howard et al.[3] in the study of immune responsiveness to pneumococcal polysaccharide (SIII) in high and low responder mice. High responders require a far smaller dose of SIII to elicit an immune response and a larger dose to be rendered tolerant than low responders. These results indicate that both immune responsiveness and tolerance are subject to the same type of genetic control.

The different susceptibility of high and low responder mice to antigen stimulation is also revealed by the duration of the ascending phase of the immune

response, which lasts about 2 wk in high responders whereas in low responders the titer of serum agglutinins drops rapidly after the 4th day (Fig. 4). This suggests a quicker inactivation of the antigen in the low than in the high responder mice as did the considerably improved responsiveness of low responder mice (Fig. 4) after repeated antigen injections. It has been shown in conventional mice that the antigen stimulation produced by intravenous immunization with SE or other particulate antigens is of short duration. The rapid phase of immune response is interrupted on the 4th–5th day postimmunization by lack of continuing antigen stimulation. The response may, in fact, be prolonged by repeating the antigen injection until other physiological factors intervene in limiting the immune response in vivo (12, 33). Prolonged stimulation of the regional lymph nodes is also produced when SE emulsified in complete Freund's adjuvants are injected into the footpads, due to the local persistence of antigen. Low line mice show a delayed but persistent agglutinin production (Fig. 6). The interline difference at peak levels is only 10-fold compared with over 100-fold after a single intravenous immunization (Fig. 4). This difference related to the route of immunization is attributable to the persistence of antigenic stimulation rather than to any biological effect of the *Mycobacterium* contained in complete Freund's adjuvant. Results similar to those presented in Fig. 6 were obtained after immunization in the footpads with SE in incomplete Freund's adjuvant. Moreover, the interline difference in agglutinin production after intravenous immunization is greatly amplified (Fig. 5) by the adjuvant effect of *C. parvum* acting directly on the spleen cells.

At the end of the ascending phase of immune response after intravenous immunization, the RFC are localized only in the spleen while after immunization in the hind footpads the number of RFC is increased only in the popliteal and inguinal lymph nodes. The interline difference in immune responsiveness observed after intravenous as well as subcutaneous immunization (Figs. 4 and 6) shows that the genetic selection operates at the level of both spleen and lymph nodes.

The above mentioned difference in susceptibility to antigen stimulation between high and low responder mice could result from modifications in the efficiency of phagocytosis and/or processing of the antigen by macrophage or in the responsiveness of the antibody-forming cells themselves. Eventually both steps of the immune response could have been modified by the selective breeding. The importance of the role of macrophages in immune response is not universal, in fact, it depends largely on the nature of the antigen (34, 35). As far as SE are concerned the data are conflicting. In vitro studies indicate that macrophages may be essential for initiating the immune response (36–38), while in vivo experiments lead to a contrary conclusion (39, 40).

Unpublished experiments on the phagocytic function of reticuloendothelial macrophages measured by the rate of blood clearance of colloidal carbon (41) showed no difference between high and low responder mice. Moreover, radio-

labeled SE injected intravenously in high and low responder mice were phagocy-tized at the same rate. After blood clearance the distribution of SE between liver and spleen macrophages was similar in the two lines. If any modification in macrophage activity is produced by the selective breeding it will concern the antigen processing rather than the antigen phagocytosis.

The role of macrophages in the different immune responsiveness of the two lines is not yet clarified. Additional unpublished findings by Howard et al.[4] on the rate of phagocytosis of SIII-^{14}C and on inactivation of T_4 bacteriophages by macrophages indicate that the functions of these cells have been modified by selective breeding. Such a modification in antigen processing and/or degra-dation could be responsible for the different immune responsiveness of the two lines. Other findings, however, indicate that the two lines differ at the level of immunocompetent cells. The allotypes of immunoglobulins are different in the two lines (7) and it has also been shown in F_2 interline hybrids that the titer of agglutinins is significantly correlated with allotypes.[6] Moreover, the two lines differ markedly in their response to pneumococcal polysaccharide in spite of the fact that macrophages do not play an essential role in the immune response to this antigen (42, 43). Until additional information is obtained, the question whether the two lines differ at the level of immunocytes or macro-phages (or both) should be left open. In the first hypothesis the group of genes segregated in each line would regulate directly the life cycle of immunocytes, while in the second hypothesis such a regulation would result indirectly from genetic modification of macrophage functions.

All data obtained from dynamic studies at the humoral level and at the cellular level by both rosette and plaque methods converge on the conclusion that the interline difference at the peak of the response results from a different tempo in antibody synthesis. The study reported in this article (Figs. 8 and 9) covers only the exponential phase during which the greatest part of the interline difference in immune responsiveness is expressed (Fig. 2). This phase corre-sponds with the rapid increase in the total number of spleen cells which is probably due to a process of cell multiplication (Fig. 7).

The data presented in Table I show that the rate of the immune response at both humoral and cellular levels is about twofold higher in the high than in the low responder mice. This estimation is confirmed by the more precise cyto-dynamic study represented in Fig. 10.

There is a good agreement between the doubling time of humoral antibody and the corrected doubling time of RFC (Fig. 10), while the doubling time of PFC is considerably shorter. Nevertheless, the interline difference in the rate of the response measured in terms of PFC is similar to that established by the other parameters (Table I). It was originally observed by Jerne et al. (44) that the exponential rise of PFC in the spleen of mice immunized with SE occurred at a higher rate than the concomittant increase of serum hemolysin. In con-

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[6] Lieberman, R. Personal communication.

ventional mice the doubling time of PFC is 6-7 hr (44, 45), while that of RFC is 12-13 hr (12), a difference which has been repeatedly confirmed (46-48). The generation time of PFC studied by thymidine-^{3}H incorporation is about 13 hr, while the doubling time is considerably shorter (6-7 hr) (49). The doubling time of RFC (about 12 hr) therefore corresponds to the multiplication time of the cell population engaged in antibody synthesis (50-52). The shorter doubling time of PFC could result from a rapid process of cell differentiation inducing into the secretory phase a small proportion of the population of RFC which multiplies every 12-13 hr after antigen stimulation.

The great majority of PFC are antibody-secreting cells of bone marrow origin (plasma cells) while the majority of rosettes are formed by lymphocytes (16, 53). Antigen-binding cells (RFC) include a large proportion of nonsecreting cells many of which are of thymus origin (28, 30, 54). This difference in origin and function of PFC and RFC explains why the interline difference is greater as measured in terms of humoral antibodies or antibody-secreting cells (PFC and plasma RFC) than in terms of total RFC (Tables I and II). It has been, in fact, demonstrated that the selective breeding affects only bone marrow–derived antibody-producing cells, while the potentiality of thymus-derived lymphocytes is not modified (4).

The results shown in Fig. 10 indicate that high and low responder mice differ in the rate of multiplication of the population of cells induced into immune response by antigen stimulation while the duration of exponential rise and the number of cells initially stimulated by antigen (target cells) are similar in both lines.

The background levels of natural RFC and antibody are also the same in high and low responder mice. Cytodynamic studies indicate that only a fraction of natural RFC (about 10%) are the receptor-bearing antigen-sensitive cells initiating the response to SE (12, 18, 55-57) through a phenomenon of antigen recognition. The other portion of natural RFC are responsible for the synthesis of natural or cross-reacting antibodies (10, 58).

The large majority of natural rosettes in high and low responder mice as well as in conventional mice are formed by small lymphocytes (16, 53, 59). After antigen stimulation other types of RFC appear rapidly: medium-sized lymphocytes, large lymphocytes, blast cells, and plasma cells (15, 16, 53, 59, 60). These different cell types increase at similar rates during the exponential phase of the response, suggesting their interdependence (53). Moreover, a morphological transition between lymphocytic and plasmocytic series has also been described (16, 61). Recently, the derivation of plasma cells from small lymphocytes has been demonstrated unequivocally (62, 63). Therefore, the immune response consists essentially in a continuous process of cell multiplication and differentiation induced by antigen stimulation acting initially on the small lymphocyte. The data presented here indicate that both processes are modified in high and in low responder mice.

The results presented in this article are compatible with the hypothesis that

the group of genes segregated in each line by selective breeding regulates directly or indirectly the rate of multiplication and differentiation of antibody-producing cells after antigen stimulation. Quantitative studies with both plaque and rosette techniques indicate that the number of cells engaged in specific antibody synthesis represents only a small percentage (about 3%) of the over-all increase in the number of spleen cells induced by antigen stimulation (12, 64).

This phenomenon is also observed in high and in low responder mice where the contribution of RFC to the total increase in the number of spleen cells is respectively 1.5 and 0.5 % (Fig. 7 and Table II). The marked interline difference in spleen enlargement produced by stimulation is therefore principally contributed by cells not actually engaged in the specific response to SE, but very probably producing immunoglobulins of other specificities. This would explain the difference in serum level of immunoglobulins found at the peak of the response against SE (7). These findings clearly suggest that the group of genes segregated in each line regulates the life cycle and differentiation of the cell population responsible for general immunoglobulin synthesis irrespective of their specificity. This interpretation would also explain the difference in responsiveness of high and low responder mice to many unrelated immunogens.

SUMMARY

Two lines of mice have been separated by selective breeding for the character "agglutinin production to heterologous erythrocytes." Around the 18th generation of selection the two lines could be considered as homozygous for the character investigated. This trait is under the control of a group of additive genes. The interline difference in the production of anti-SE agglutinins was verified for the range of antigen doses from subimmunogenic to maximal. After intravenous immunization with an optimal dose of SE, the duration of the exponential rise in serum antibody was 4–5 days in both lines. At this time most of the interline difference in responsiveness is already expressed. A cytodynamic study carried out in terms of plaque-forming cells (PFC) and rosette-forming cells (RFC) in the spleen during the exponential phase showed that the principal interline difference is found in the doubling time of cells engaged in the immune response.

More precise cytodynamic analysis made in terms of RFC showed that the doubling time of RFC is 9 hr in high responder and 16 hr in low responder mice. The duration of the exponential rise and the number of target cells stimulated by antigen is the same in both lines. The interline difference at the end of the exponential rise (4 days postimmunization) is larger in terms of serum antibody (30–40-fold) than in terms of PFC or RFC (20- and 11-fold, respectively).

A morphological study of RFC in nonimmunized mice showed that about 90% of rosettes were formed by small lymphocytes in both lines. The remainder were medium-sized lymphocytes. At the peak of the cellular response the RFC have differentiated into large lymphocytes, blast cells, and plasma

cells. The contribution of plasma cells to RFC is much greater in the high than in the low line. The cytodynamic and morphologic results presented in this article are compatible with the hypothesis that the group of genes segregated in each line during the selective breeding control and regulate the rate of multiplication and differentiation of the antibody-producing cells.

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