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Immunocytochemical Studies on B Lymphocytes from the Biozzi High and Low Lines of Mice Selected for Their Responses to Heterologous Erythrocytes[1] (37990)

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The high and low antibody producing lines of mice were developed by Biozzi and collaborators by breeding outbred Swiss albino mice on the basis of their agglutinin response to foreign red cells (1). (These lines are here referred to as "high" and "low" lines.) After more than 16 generations of selective breeding, two lines of mice were obtained differing markedly in the humoral immune response not only to the antigen used in the selection process, sheep and pigeon red blood cells, but also to unrelated ones such as ovalbumin, pneumococcal polysaccharide, hemocyanin, etc. (2–4). Studies have been carried out with these lines of mice in an attempt to explain the nature of their widely divergent immune responses as well as to determine the cellular basis of the genes concerned. Biozzi and collaborators concluded on the basis of kinetic studies of the cellular anti-sheep red blood cell response that the number of progenitors of antibody forming cells was the same for both lines, the differences being the rate of cell proliferation following antigen stimulation (3, 5). In addition, low line mice were found to be hypogammaglobulinemic for every immunoglobulin (Ig) class whereas the high line mice had normal Ig levels in their serum; the differences in the level of serum Ig became more marked following antigenic stimulation (6). These observations, added to the fact that cell mediated immune reactions were identical in both lines (7), suggested that the genetic differences were somatically expressed primarily at the level of B lymphocytes. However, differences in the handling of pneumococcal polysaccharide by both lines have been observed recently (4), and this has raised the possibility that macrophage function may have been affected by the selection. This was confirmed by experiments demonstrating that antigens of sheep erythrocytes persist much longer in immunogenic form in the spleen of "high" responders than in that of *low* responders (8). These findings are not expected considering the well known interrelationships among different cellular functions in immunity.

In this study we have investigated the B cell populations in the Biozzi lines, using immunocytological methods. We were interested in determining whether there were differences in the number of B lymphocytes in lymphoid organs or in the amount, distribution and behavior of the surface Ig on the B cell.

Materials and Methods. Mice. The high and low line mice were derived from the lines originally developed by G. Biozzi in Paris. The mice were first sent to Dr. R. Asofsky at the National Institutes of Health (U.S.A.); at the time of shipment to this country the selection for high and low antibody responses has proceeded for 20 generations. The mice have been bred at Harvard in a random manner avoiding brother-to-sister mating. Mice were maintained on regulator laboratory food and on

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chlorinated water, and have not shown any overt evidence of disease.

Immunocytological Analyses. Cells were harvested from spleen, thymus, and lymph nodes by regular procedures using Hanks' balanced salt solution (Microbiological Associates, Bethesda, Maryland) containing 1% fetal calf serum (Associated Biomedics, Buffalo, New York). The presence of Ig on the surface of such cells was investigated using the fluorescent antibody method as adapted for cell membrane fluorescence. The antisera was a polyvalent rabbit anti mouse Ig—in previous studies (9) the specificity and control tests have been detailed. Five million cells were incubated with 20 μg of the fluorescent anti Ig (Fluorescein: Protein ratio of 4.1), in a total volume of 50–100 μl at 2°–4°C for 30 min, then spun, washed three times, and examined under the fluorescent microscope. For fluorescence microscopy, we used a Zeiss microscope employing a FITC primary filter manufactured by Optisk Laboratorium, Lyngby, Denmark. The redistribution of surface Ig after complexing with the fluorescent anti Ig was studied by examining microscopically samples of cell suspension (containing 5×10^6 cells in 0.5 ml) after incubation at 37°C for different times up to 15 min. In one experiment the cells were stained with a purified fluorescent anti μ serum obtained from Dr. H. M. Grey.

Antigen Binding Cells. The number of cells in spleens capable of binding antigen was studied by autoradiography employing the hemocyanin from Megathura Crenulata (keyhole limpet hemocyanin, KLH). KLH was purified by ultracentrifugation, and labeled with ^{125}I to a specific activity of 17 μCi per μg (10). Ten million spleen cells were incubated with 20 μg of ^{125}I KLH in a volume of 200 μl for 40 min at 4°C; the cells were smeared on gelatin-coated slides after washing them 5 times with large volumes of media. Slides were emulsed with Kodak NTB 2 liquid emulsion, developed after 2 weeks of incubation and stained with Giemsa. All cells containing more than 10 grains were scored as positive; about 50,000 were counted.

Quantitation of Surface Ig. Surface Ig was quantitated by an antigen inhibition test using mouse Fab as antigen. The procedure has been detailed well in previous studies (11–12). Various amounts of spleen cells are incubated in a solution of ^{125}I Fab, anti Fab; the extent to which the surface Ig of the spleen cells blocks the binding of Fab to the antibody is estimated and compared to the inhibition produced by soluble Ig. The following technique was followed in the present experiments. Mouse Fab was labeled with ^{125}I to a specific activity of about 1 μCi per μg. For the *standard inhibition curve,* 500 μl of an appropriate dilution of a polyvalent rabbit anti mouse Ig were incubated with 100–500 ng of a pool of mouse IgG in 500 μl at 4°C with constant stirring for 18 hr. The tubes were spun and from them 3 aliquots of 80 μl were separated and then incubated with 5 ng of ^{125}I Fab (in 20 μl) for 1 hr at 4°C; the reaction was stopped by addition of 100 μl of 95% saturated ammonium sulphate (13); the precipitates were spun, washed once with 47.5% ammonium sulphate solution and counted in a Packard gamma ray Spectrometer. For the *test* 5×10^7 to 10^7 spleen cells (in 500 μl) were incubated with the anti Ig antibody (in 500 μl) for 18 hr, then spun and the supernatant assayed, in triplicate, as above—that is 80 μl were incubated with 5 ng of ^{125}I Fab. In every procedure the appropriate standard and control were included (11–13). The dilution of anti Ig used in the test precipitated about 30% of the labeled Fab. Two special precautions must be taken in the calculations: one must refer the results obtained to the standard inhibition curve only in the area of strict proportionality between amount of antigen employed and degree of inhibition; also the amounts of cells to be referred to the standard curve must be in the range where amounts of cells inhibit proportionally the standard reaction. In each experiment the number of B cells was estimated by immunofluorescence. The contents of surface Ig was expressed as ng per 10^7 Ig positive cells.

Immune Response to KLH. We followed methods previously described (14). Eight-week-old mice were immunized with 1 μg of soluble KLH intraperitoneally; 3 weeks later

TABLE I. B Lymphocytes in Spleens of High and Low Lines.[a]

Line	Total number ($\times 10^{-6}$)	B lymphocytes (Ig positive) (%)	B lymphocytes (Ig positive) ($\times 10^{-6}$)	Ig per B cells (ng per 10^7)
		8–9 weeks of age		
High	287.2 (25.09)	43.8 (2.2)	120.8 (10.9)	264.3 (27.8)
Low	232.4 (21.15)	46.1 (1.8)	91.1 (16.4)	178.3 (25.3)
				[$P < 0.05$]
		13–14 weeks of age		
High	276.9 (13.1)	44.0 (1.4)	122.1 (6.9)	320.7 (56.8)
Low	181.7 (13.1)	47.2 (1.9)	85.3 (6.4)	255.8 (47.1)
	[$P < 0.01$]		[$P < 0.01$]	

[a] Each group compares 7–8 mice. Figures are arithmetic means; figures in parenthesis represent standard error of the mean. Each group was subjected to statistical analysis by Student's t test. The P values of those groups with statistical difference are indicated.

they were challenged with 50 μg intraperitoneally and were then bled for quantitation of serum anti KLH 7 and 14 days later. Anti KLH antibodies were quantitated by a Farr method (14).

Results. Immunocytological analysis of lymphoid tissues. High and low lines of mice were examined for the content of B lymphocytes, i.e., those with easily detectable surface Ig molecules, in the spleen, lymph nodes and thymus. Seven to eight mice of each line comprising both sexes were examined when 8–9 weeks of age or 13–14 weeks. The results of these two age groups have been separated. Table I and Fig. 1 show the experimental results obtained with spleen cells. The *percentage* of Ig positive lymphocytes was the same in both lines and for both age groups. However, in absolute numbers there was a clear tendency for high line mice to contain more cells in their spleen, and hence, the total number of Ig positive lymphocytes was higher in the high line. Such differences in absolute number of Ig positive lymphocytes between high and low lines were not significant in the young age group (by Students t test the P value was <0.20 and >0.10); the differences were highly significant in the older age group ($0.01 < P > 0.001$). The number of Ig positive lymphocytes in lymph nodes of 4 mice from each line of the old age group was similar (Table II). Thymus of both strains contained less than 1% Ig positive cells (Table II).

The pattern of distribution of Ig on the surface of lymphocytes from both lines was identical. The distribution was judged by the immunofluorescent pattern of Ig immediately after the reaction of the cells with the fluorescent anti Ig at 4°C. It consisted of a finely stippled distribution throughout the entire cell surface. The redistribution of the anti Ig–Ig complex on the surface of the lymphocytes was studied by incubating the suspension of lymphocytes—following their

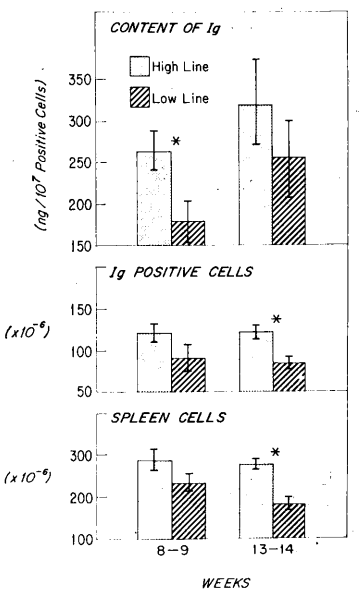

FIG. 1. These are the same results of Table I, but presented in graphic form. Asterisk refers to differences that are statistically different.

TABLE II. B Lymphocytes in Lymph Nodes and Thymus.[a]

Line	Lymph nodes			Thymus		
	Total cells ($\times 10^{-6}$)	Ig positive		Total cells ($\times 10^{-6}$)	Ig positive	
		%	($\times 10^{-6}$)		%	($\times 10^{-6}$)
High	11.0	30.4	3.3	72.5	0.2	0.15
High	13.8	32.6	4.5	54.0	1.4	0.76
High	19.2	27.9	5.4	106.5	0.4	0.43
Low	16.4	36.3	6.0	108.5	0.8	0.87
Low	16.6	25.7	4.3	104.5	0.5	0.52
Low	18.6	24.4	4.5	80.0	0.4	0.32

[a] 13–14 weeks of age—Table shows results of 3 individual mice from each strain.

reaction with fluorescent anti Ig at 4°C—at 37°C for various periods of time. In normal murine lymphocytes the complexes usually are displaced into one pole of the cell—the cap—and then are interiorized in small vesicles. The redistribution phenomena was the same in lymphocytes from both lines. Most lymphocytes showed the complexes in a cap within the first 5 min at 37°C and then in small vesicles.

Detailed morphological analysis of smears of spleen cells stained with Giemsa was performed on 3 mice from each strain of the older age group. These results, shown in Table III, do not indicate marked differences in the percentage of different lymphoid cells between both groups.

The percentage of spleen lymphocytes binding ^{125}I KLH was identical in both strains: 0.07, 0.09 and 0.11 for the three high line mice studied, and 0.08, 0.09 and 0.12 for the low line. The study was done in mice of the older age group.

Content of Surface Ig. Lymphocytes from both strains were studied for their content of surface Ig by the inhibition test using ^{125}I labeled mouse Fab. In our hands the content of surface Ig in various inbred strains of mice ranges about 250–300 ng per 10^7 Ig positive spleen cells which calculates to about 10^5 molecules per individual (assuming that the Ig on the surface is a monomer of 140,000 molecular weight). In the young age group the low line lymphocytes contained less surface Ig than the high line (Table I, Fig. 1). At this age, cells from high line contained 264 ng of Ig per 10^7 cells which calculates to about 98,000 molecules per individual cell, while the cells from low line contained 178 ng per 10^7 or 66,000 molecules per cell. These differences by Students t test are significant with P values

TABLE III. Cell Types in Spleen.[a]

	High line			Low line		
	1	2	3	1	2	3
Lymphocytes	84.5	87.7	89.6	81.0	77.5	82.6
Plasmacells & blast cells	2.4	4.3	5.2	2.7	2.0	1.3
Monocyte-macrophages	5.0	3.1	1.7	5.0	5.0	2.2
Erythroid series	0.5	1.4	1.7	4.0	5.0	4.8
Neutrophils	6.8	2.2	1.7	6.6	10.0	8.3
Eosinophils	0.8	1.2	0.0	0.7	0.5	0.8

[a] This table shows cytological analysis of Giemsa stained smears of 6 individual mice. About 300 cells were counted; figures represent percentage.

of <0.05 and >0.02. The differences between both lines disappeared in the older age group: high line exhibited 321 ng/10^7 cells or 118,400 molecules per cell; low lines exhibit 256 ng/10^7 cells or 95,000 molecules per cell.

Immune Response to KLH. In order to confirm the previous results of Biozzi within our colony, we studied the immune response to KLH in mice 12 weeks of age. Table IV shows the results. Clearly, the four high line mice studied made a good anti KLH immune response, while all the four low mice responded poorly.

Immunocytological analysis was made of the spleen of such immunized mice following the parameters described in the experiments with nonimmunized mice. Prior to studying the cells, all 4 mice of each line were also immunized, this time with 10^8 sheep red cells a week prior to harvesting the cells. The results are shown in Table V. Except for one, the low line mice contained a smaller number of Ig positive lymphocytes in their spleen than the high line; note also that 3 out of 4 of the high line mice contained more surface Ig per Ig positive cell than the four low line mice.

Discussion. The high and low line of Biozzi mice bred at Harvard show a marked difference in their antibody response to a protein antigen such as KLH in agreement with the data previously reported for the original lines bred in France (2). Several points of interest emerge from our present study. Clearly, the relative distribution of B and T lymphocytes—as judged by the presence or absence by immunofluorescence of surface Ig—and the percentage of antigen binding cells was the same for both lines. Furthermore, the surface distribution of Ig as well as the redistribution of anti Ig–Ig complexes on the surfaces was identical in both strains. The differences that are observed relate to: (a) the mean content of surface Ig per individual B lymphocyte; and (b) the absolute number of splenic B lymphocytes.

The B cells from the low line exhibited about one-third less surface Ig than that of the high line which had amounts comparable to that seen in inbred strains of mice. These differences were observed in the young age group at a time when the concentration of serum Ig is also quite different—about one-half less in the low line. However, as the mouse aged, the differences in content of surface Ig disappeared. These differences in content of surface Ig indicate that there must be factors which influence, control or regulate the content of surface Ig. The observed differences could be related to the half life of surface Ig, or the amounts synthesized by the cell, any one of which may (or may not) be influenced by genetic or environmental factors. It is obvious that further study needs to be done in order to explain the observation.

The second difference that was observed relates to the absolute amount of B lymphocytes in spleens at a time relatively late in life. Because of the late onset, this probably is an indication of the environmental influences on the different immune systems.

TABLE IV. Anti-KLH Immune Response.[a]

	Day 7		Day 14	
Line	% KLH bd 1:2	ABC-33	% KLH bd 1:2	ABC-33
High	29.6 (1.39)	1.6 (2.0)	20.7 (1.2)	0.83 (1.5)
Low	0.6 (2.3)	—	1.0 (2.7)	—

[a] This table shows the anti-KLH immune response of 4 mice of each line. Figures represent geometric means; parenthesis refer to standard error of the mean. Titers of anti-KLH were obtained 1 and 2 weeks after secondary challenge with 50 μg of KLH intraperitoneally; mice were primed 3 weeks prior to challenge with 1 μg of KLH. The table shows each time, the percentage of KLH bound at a 1:2 serum dilution and wherever possible, the ABC-33 value. Statistical evaluation by Student's t test shows P values of <0.01 and >0.001 at each time.

TABLE V. B Lymphocytes in Spleens of Immunized Mice.[a]

Line	Total number ($\times 10^{-6}$)	B lymphocytes (Ig positive) %	B lymphocytes (Ig positive) ($\times 10^{-6}$)	Ig per B cells (ng per 10^7)
High	296	64.5	191	281
	300	62.0	186	347
	224	57.5	129	442
	217	56.8	123	502
	(259.2)	(60.2)	(157.2)	(393.0)
Low	300	57.6	173	286
	235	48.5	114	258
	155	46.5	72	269
	150	47.9	71	296
	(210.0)	(50.2)	(107.6)	(277.3)

[a] These 4 mice were the same immunized with KLH (Table IV); they were challenged with sheep red cells intraperitoneally and examined cytologically a week later.

The high lines being more responsive will be expected to have more lymphocytes with immunological memory to the environmental antigens.

Finally, the question arises whether the subtle changes that have been found account for the marked differences in antibody synthesis. It is most likely that this is not the case. Previous studies using sheep erythrocytes demonstrated a similar number of antigen binding cells (i.e., rosette-forming cells) in spleens of nonimmunized mice from both strains. Four days after immunization the number of rosette-forming cells increased about tenfold more in high line than in low line mice. At the same time the number of plaque forming cells in the high line was twentyfold higher than in the low line mice. These findings suggested that the major interline difference concerned the rate of multiplication and differentiation of the clone of specific immunocompetent lymphocytes. The overall results in this report would support the previous finding. The most likely hypothesis to explain the differences in general immune responsiveness of these two lines is that the genetic control is exerted at the some step in the regulation of cell proliferation and differentiation.

Summary. The high and low antibody producing lines of mice developed by Biozzi were studied immunocytochemically in an attempt to determine several parameters of B lymphocyte function. At 8–9 weeks of age, both lines contain the same number of B cells in spleen, lymph node and thymus. The B cells of the high line contain about one-third more surface Ig; the distribution of surface Ig in cells of both lines as well as the redistribution of the surface Ig–anti Ig complexes were identical. At 13–14 weeks of age, the number of B cells was higher in the high antibody producing mice; at this age, however, the mean content of Ig per B lymphocyte was identical.

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1. Biozzi, G., Stiffel, C., Mouton, D., Bouthillier, Y., and Decreusefond, C., *in* "Protides of Biological Fluids," Pergamon Press, Oxford, 1970.

2. Biozzi, G., Stiffel, C., Mouton, D., Bouthillier, Y., and Decreusefond, D., Progr. Immunol. **1**, 529 (1972).

3. Biozzi, G., Stiffel, C., Mouton, D., Bouthillier, Y., and Decreusefond, D., J. Exp. Med. **135**, 1071 (1972).

4. Howard, J. G., Christie, B. M., Couttenay, B. M., and Biozzi, G., Eur. J. Immunol. **2**, 269 (1972).

5. Biozzi, G., Stiffel, C., Mouton, D., Bouthillier, Y., and Decreusefond, C., Transplant. Proc. **4**, 339 (1972).

6. Biozzi, G., Asofsky, R., Lieberman, R., Stiffel, C., Mouton, D., and Benacerraf, B., **132**,

752 (1970).

7. Liacopoulos-Briot, M., Bouthillier, Y., Mouton, D., Lambert, F., Decreusefond, C., Stiffel, C., and Biozzi, G., Transplantation **14**, 590 (1972).

8. Unanue, E. R., Perkins, W. D., and Karnovsky, M. J., J. Exp. Med. **136**, 885 (1972).

9. Greenwood, F. C., Hunter, W. M., and Glover, J. S., Biochem. J. **89**, 114 (1963).

10. Rabellino, E., Colon, S., Grey, H. M., and Unanue, E. R., J. Exp. Med. **133**, 156 (1971).

11. Grey, H. M., Colon, S., Campbell, P., and Rabellino, E., J. Immunol. **109**, 776 (1972).

12. Lerner, R. A., McConakey, P. J., Jansen, I., and Dixon, F. J., J. Exp. Med. **135**, 136 (1972).

13. Unanue, E. R., and Cerottini, J.-C., J. Exp. Med. **131**, 711 (1970).

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