DENSITY OF SURFACE IMMUNOGLOBULIN AND CAPPING ON RAT B LYMPHOCYTES

I. Changes with Aging*

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The immune vigor of the aging animal is diminished in comparison with young animals (1, 2) and the diminution of immune function in old animals has been attributed to dysfunction of T cells (3, 4), B cells (4, 5), and/or their subsets. Immune deficiencies of T and/or B lymphocytes in aged animals have not yet been explained at a cellular or molecular level. Most evidence indicates that there is no significant loss in cell number of either of the classes of lymphocytes. We have postulated that there is a loss of surface immunoglobulin (SIg)[1] on the B cells of aging animals and this loss might occur as a result of decreased densities of SIg on individual B lymphocytes or a compositional change in subsets of B cells that carry varying densities of SIg.

Materials and Methods

Rats. Young adult male and female Lewis rats, 12–16 wk old, and aging male and female Lewis rats were obtained from the breeding colony of the Research Institute of the Scripps Clinic and maintained in a light- and temperature-controlled environment. They were allowed food ad libitum. The median age of survival for Lewis rats in our colony is 24 mo. Rats that were 24 mo or older were considered old rats in this study.

Cells. Rats were killed by exsanguination under ether anesthesia. Spleen cell suspensions were prepared by teasing apart spleens in minimum essential medium (MEM) and filtering through nylon mesh. Mononuclear cells were isolated by centrifugation on a Ficoll-Hypaque gradient (Ficoll, Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.; Hypaque, Winthrop Laboratories, N. Y.). The interface cells were washed three times in MEM and adjusted to a concentration of 3×10^7 cells in MEM supplemented with 10% fetal bovine serum (FBS) (Flow Laboratories, Inc., Rockville, Md.). The viability of the cells was 96–99% as determined by trypan blue exclusion. B cells were identified by staining whole spleen cell suspensions with fluorescein-labeled antibodies (see below).

Antibodies. The IgG fraction of a polyvalent goat antiserum to rat immunoglobulin (GARG) and of a rabbit antiserum to goat IgG (RAGG) were isolated by precipitation with 50% ammonium sulfate and by chromatography of the precipitate dissolved and equilibrated in

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[1] *Abbreviations used in this paper:* SIg, surface immunoglobulin; MEM, minimum essential medium; FBS, fetal bovine serum; GARG, goat antibody to rat immunoglobulin; RAGG, rabbit antibody to goat IgG; FITC, fluorescein isothiocyanate; EBSS, Earle's balanced salt solution; BSA, bovine serum albumin; FACS, fluorescence-activated cell sorter.

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0.01 M phosphate buffer, pH 8.0, on a DE-52 column (Whatman, Inc., Clifton, N. J.). $F(ab')_2$ fragments were prepared by digesting goat IgG at pH 3.8 and rabbit IgG at pH 4.0 with 3% pepsin, wt/wt, (Worthington Biochemical Corp., Freehold, N. J.) for 18 h at 37°C. Immunoelectrophoresis (1.2% noble agar in pH 8.6 barbital buffer) of the pepsin-digested molecules showed a single band which migrated slower than intact IgG. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the pepsin digests did not show undigested IgG.

Fluorescein isothiocyanate (FITC) (BioQuest, BBL, & Falcon Products, Becton, Dickinson, & Co., Cockeysville, Md.) in carbonate buffer at pH 9.5 was conjugated to F(ab')2 fragments of GARG and RAGG by dialysis for 24 h at 4°C (0.1 mg FITC/mg $F(ab')_2$) Free fluorescein was removed by dialysis and conjugates with a fluorescein/protein ratio of 4–6:1 were isolated by chromatography on a DE-52 column. The antibodies, at optimal concentration, stained 23% of splenic lymphocytes from young rats and 15% of splenic lymphocytes from old rats; and <0.2% of rat thymocytes; indicating that the antisera did not react with species specific antigens present on thymocytes.

Capping Procedure. 3×10^6 cells, suspended in 0.1 ml of MEM-FBS in 12 × 75 mm polystyrene tubes (BioQuest, BBL, & Falcon Products, Becton, Dickinson, & Co.) were mixed with 25 μg of $F(ab')_2$--GARG and kept on ice for 30 min. The cells were washed two times, resuspended in 50 μl MEM-FBS, mixed with 25 μg of $F(ab')_2$--RAGG--FITC, kept on ice for 30 min, washed two times, resuspended in 0.5 ml of MEM-FBS, and incubated at 20°C or 37°C for varying periods of time. The reaction was stopped by the addition of 100 μl of 4% paraformaldehyde yielding a final concentration of 0.7%. In selected experiments, cells were capped directly with $F(ab')_2$--GARG--FITC. Cells were examined with a Zeiss fluorescence microscope (Carl Zeiss, Inc., N. Y.). 100 Fluorescent cells were scored for each determination. Cells with less than one-third of the membrane positively stained were considered capped. The percentage of capped cells was calculated as the fraction of capped cells over total fluorescent cells times 100. The percentage of positively stained lymphocytes was also determined at each time point. The loss of positively stained cells was determined by the following formula:

$$\% \text{ loss of fluorescent cells} = \frac{\text{stained cells, time 0} - \text{stained cells, time t}}{\text{stained cells, time 0}} \times 100.$$

In some experiments, cells were preincubated in 1×10^{-5} M colchicine (Sigma Chemical Co., St. Louis, Mo.) for 60 min at 37°C and then treated as outlined above.

Flow Microfluorimetry. 3×10^6 cells in 0.1 ml of Earle's balanced salt solution (EBSS) (Flow Laboratories, Inc., Rockville, Md.), 2% bovine serum albumin (BSA) (Miles Laboratories Inc., Miles Research Products, Elkhardt, Ind.), and 0.1% NaN_3, were incubated with $F(ab')_2$--GARG followed by $F(ab')_2$--RAGG--FITC and washed, and then analyzed in a fluorescence-activated cell sorter (FACS) (FACS II, Becton, Dickinson FACS Systems, Mountain View, Calif.). An explanation of the principle of flow microfluorimetry has been presented by Loken and Herzenberg (6). $1–2 \times 10^5$ viable cells were analyzed for light scatter (cell size) and fluorescence intensity for each determination. The data were plotted with the number of cells on the vertical axis and fluorescence channel numbers on the horizontal axis (Fig. 1). Cells with a greater amount of bound FITC-labeled antibody were present in the higher channels, i.e., cells with greatest density of SIg were detected in the higher numbered channels. The fluorescence curve encompasses channels 0–1,000. Cells in channels 80–320 were considered low density, cells in channels 320–600 intermediate density, cells in channels 600–800 high density, and cells in channels 800–1,000 very high density. The percentage of cells under each portion of the curve was determined as follows: (cells in specified channels)/(total scatter [cell number] × 100.

Results

Cap Formation. When splenic lymphocytes were incubated at 0°C with $F(ab')_2$--GARG, followed by $F(ab')_2$--RAGG--FITC, and then kept at 37°C for varying time periods, the B cells of young and old Lewis rats capped at different rates (Table I). The difference was statistically significant at 2 min ($P < 0.02$) and was maintained throughout the observation period. At 20°C, capping proceeded slower and the difference in capping rate was more evident (Table I). A comparison of the capping

TABLE I

*Percentage of Fluorescent Cells Capped**

Time	37°C			20°C		
	Young Lewis rats	Old Lewis rats	P‡	Young Lewis rats	Old Lewis rats	P‡
min	%	%		%	%	
2	22 ± 1 (4)§	14 ± 1 (4)	<0.02	7 ± 1 (6)	5 ± 1 (6)	NS
5	30 ± 2 (13)	24 ± 2 (16)	<0.05	15 ± 2 (6)	9 ± 2 (6)	<0.05
15	49 ± 2 (17)	34 ± 2 (19)	<0.001	30 ± 3 (6)	18 ± 2 (6)	<0.01
30	69 ± 2 (10)	60 ± 3 (12)	<0.02	50 ± 2 (6)	29 ± 2 (6)	<0.001
45	—	—		68 ± 2 (6)	49 ± 1 (6)	<0.001

* Data shown are the mean ± SEM. 3×10^6 spleen cells in MEM-FCS were incubated with $F(ab')_2$- -GARG and stained by $F(ab')_2$- -RAGG- -FITC and kept at 20°C for 37°C for 2–45 min. The percentage of capped cells = (capped cells)/(total fluorescent cells) × 100.

‡ *P* value was calculated by Student's *t* test and compares capped and noncapped cells.

§ In parentheses number of trials; in each trial 100 cells were counted.

TABLE II

*Percent Loss of Fluorescent Cells**

Time	Young Lewis rats	Old Lewis rats	P‡
min	%	%	
2	22 ± 3 (4)§	8 ± 1 (4)	<0.01
5	34 ± 2 (9)	17 ± 2 (14)	<0.001
15	51 ± 1 (11)	31 ± 1 (18)	<0.001
30	69 ± 3 (8)	47 ± 3 (12)	<0.001

* Data shown are the mean number of stained cells ± SEM. 3×10^6 spleen cells in MEM-FBS were mixed on ice with $F(ab')_2$- -GARG followed by $F(ab')_2$- -RAGG- -FITC, and kept at 37°C for 2–30 min. % loss fluorescent cells = (stained cells, time 0 − stained cells, time ×)/(stained cells, time 0) × 100.

‡ *P* value was calculated by Student's *t* test, and compares young versus old.

§ In parentheses number of trials; in each trial 200 cells were counted.

rates at both temperatures showed that during the observation period the percentage of fluorescent cells capped was somewhat greater at 37°C.

Similar differences in capping rates were seen when cells were capped directly with $F(ab')_2$--GARG--FITC and there was no significant difference in cap number between cells capped with the direct or indirect technique (data not shown).

Shedding and Endocytosis. The visible effects of the capping process terminates in endocytosis and/or shedding of the cross-linked receptors (7–9). Observation of the capping process showed that as the number and percentage of cells with caps increased, the number of all stained cells (rings and caps) decreased. We measured the rate of shedding/endocytosis by counting the number of fluorescent cells at 0 time and at serial time intervals thereafter (Table II). Shedding and/or interiorization of the cross-linked receptors occurred quickly, whereas the percentage of capped lymphocytes was increasing. The percentage of fluorescent cells which lost their stain was greater in young rats than in old rats. The difference between the populations was statistically significant as early as 2 min and was greatest at 30 min (Table II). The rate of loss of stained lymphocytes was similar in both groups.

Relative Density of Surface Immunoglobulin. A possible explanation for the observations described above was that the density of SIg varied in rats of different ages. We,

TABLE III

*Fluorescence Intensity of Stained Spleen Cells**

Channel No.	Young Lewis rats	Old Lewis rats	P‡
	%	%	
80–320§	40 ± 3	50 ± 4	NS
320–600	27 ± 1	26 ± 1	NS
600–800	19 ± 1	16 ± 1	<0.05
800–1000	16 ± 1	12 ± 1	<0.01

* Data shown are the mean percentage of stained cells ± SEM for 4–10 separate experiments. 3 × 10^6 spleen cells in EBSS-BSA-NaN_3 were incubated with $F(ab')_2$- -GARG and stained by $F(ab')_2$- -RAGG- -FITC. 1–2 × 10^5 viable cells were analzyed for fluorescence intensity in a FACS-II. The data show the percentage of cells present in the specific fluoresence channels.

‡ *P* value was calculated by Student's *t* test, compares young versus old.

§ Fluorescence channels.

therefore, measured the relative density of SIg as ascertained by fluorescence intensity in the FACS.

The percentage of cells lying under various portions of the fluorescence curve was determined (Table III). The largest population of fluorescent cells were in the low density area of the curve, channels 80–320; fewer cells were present in the intermediate channels, 320–600 and high density channels, 600–800; and the smallest number of cells were in the very high density channels, 800–1,000. Analysis of the light scatter curves showed no significant difference in the size of lymphocytes from young and old animals and no significant difference in the size of lymphocytes in the low and very high density areas of the curves. Comparison of the fluorescence histograms of the young and old animals showed a significant decrease in the percentage of lymphocytes in the high (600–800) and very high density channels (800–1,000) in old rats.

Density of Surface Immunoglobulin and Capping. After ascertaining that there was a decreased number of cells with medium and high density SIg on lymphocytes from old rats, we examined the relationship of density of SIg and rate of modulation. A plausible way of doing this experiment would have been to sort out cells with different densities of SIg and determine their capping rate. However, this experiment requires that the membrane be perturbed by the binding of anti-immunoglobulin and that the cells be exposed to sodium azide for long periods of time to prevent surface movements during the sorting procedure.

To obviate these difficulties, we analyzed populations of stained splenic cells under noncapping conditions (0–4°C, 0.1% NaN_3) for distribution of cells according to stain intensity. Populations from the same pools of splenic cells were stained, allowed to cap during a 15-min period at 37°C and then placed on ice in medium containing 0.1% NaN_3. These cells were also analyzed in the FACS according to intensity of stain.

15 min after incubation under capping conditions, a proportion of fluorescent cells had shed or endocytosed their SIg (Table II). Assuming that cells which shed their SIg first also cap quickest, we analyzed the fluorescence histograms looking for a shift in the number of cells under various portions of the curve. In this way, we determined which cells had cleared their cross-linked SIg first and inferentially had capped most rapidly.

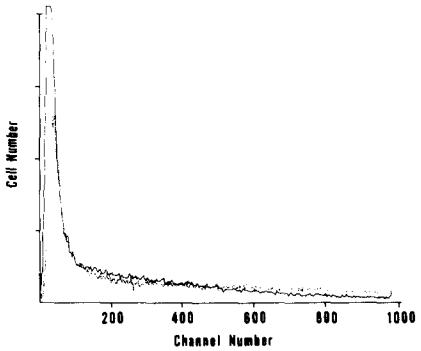

FIG. 1. Fluorescence histogram of cells from an old Lewis rat incubated with $F(ab')_2$- -GARG followed by $F(ab')_2$- -RAGG- -FITC under noncapping (-----) and capping (——) conditions. Curves represent fluorescence histograms for 2×10^5 viable cells in each population. Cell number is plotted on the vertical axis. The fluorescence channel numbers are plotted on the horizontal axis. The more intensely fluorescent cells are present in the higher numbered channels.

TABLE IV

*Fluorescence Intensity of Stained Spleen Cells**

Comparison of Populations under Capping and Noncapping Conditions

Channel number	Young Lewis rats		Old Lewis rats		Old and young Lewis rats- -combined data		
	Time 0	15 min	Time 0	15 min	Time 0	15 min.	*P*‡
	%	%	%	%	%	%	
80–320§	33 ± 1	36 ± 1	42 ± 1	48 ± 1	37 ± 3	42 ± 3	NS
320–600	30‖	35 ± 1	28 ± 1	31	29 ± 1	33 ± 1	<0.05
600–800	21 ± 1	17 ± 1	17	13 ± 1	19 ± 1	15 ± 2	NS
800–1000	17 ± 1	11 ± 2	13 ± 1	8 ± 1	15 ± 1	10 ± 1	<0.02

* Data shown are the means ± SEM for 2–4 separate experiments. 3×10^6 spleen cells were stained with $F(ab')_2$- -GARG followed by $F(ab')_2$- -RAGG- -FITC. 2×10^5 viable cells were analyzed for each determination in a FACS II. Cells analyzed at time 0 were stained under noncapping conditions (0°–4°C in EBSS-BSA-NaN3). Cells analyzed after capping were stained under capping conditions (stained on ice in MEM-FBS, kept at 37°C for 15 min). The reaction was stopped by placing the cells on ice in the presence of 0.1% NaN_3.

‡ Fluorescence channels.

§ *P* value was calculated by Student's *t* test and compares capped and noncapped cells.

‖ In experiments in which there is no SEM, experimental values were the same for all determinations.

Fig. 1 depicts such an experiment. The unbroken line shows that in a population of cells incubated under capping conditions for 15 min there is a diminished proportion of cells in the high density area of the curve (channels 600–1,000) as compared with cells stained under noncapping conditions (broken line). The divergence between the curves became evident at channel 500, and was greatest at the very high density area (800–1,000). Table IV shows a decrease in the number of cells in the brightest channels (600–1,000) suggesting that these cells had capped and shed their SIg first. This process occurred in lymphocytes from both young and old rats.

Effects of Colchicine on Cap Formation. Fig. 2 shows that pretreatment of cells with 1×10^{-5} M colchicine increased the percentage of capped lymphocytes of young rats at 37°C ($P < 0.001$) and at 20°C ($P < 0.01$). Colchicine treatment of splenic lymphocytes of old rats did not appreciably increase cap formation at either temperature and did not reverse the slow capping kinetics.

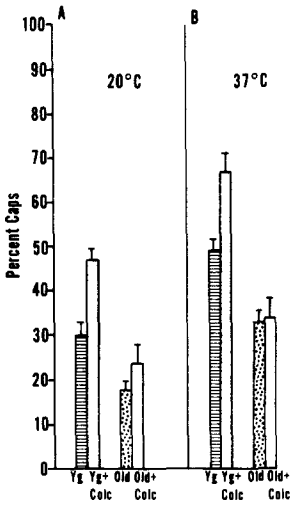

FIG. 2. Effects of colchicine on the percentage of spleen SIg positive cells capped after incubation at 20°C or 37°C for 15 min. Means ± SEM shown are for 6 separate determinations per column (A, 20°C) or 6–15 determinations per column (B, 37°C). Hatched bars, young (yg); stippled bars, young plus colchicine (yg + colc); dotted bars, old; open bars, old plus colchicine (old + colc).

Discussion

In this study we have demonstrated: (*a*) the rate of capping and shedding of cross-linked surface receptors is slower in splenic lymphocytes from old rats; (*b*) the anti-tubulin reagent, colchicine, increases the rate of capping in young animals but not in old; and (*c*) in populations of lymphocytes from old rats there are fewer cells with high density of SIg as measured in the FACS. We have also shown that after cross-linking of SIg with anti-immunoglobulin, the cells with the highest density of SIg cap and shed sooner than those cells with less SIg. The slowness of capping and shedding in aged lymphocytes may be attributed, at least in part, to a change in cell popula[illegible] fewer cells in old animals bearing dense SIg.

Modulation of surface immunoglobulin is a complex energy and temperature-dependent process which is related to density of surface molecules, interaction with cytoskeletal structures, membrane fluidity, and intracellular metabolism. The relative density of SIg is decreased on B cells of old rats. More specifically, analysis of fluorescence histograms indicates that as rats age they lose cells with a high density of SIg. Analysis of cells in the FACS under noncapping conditions (4°C, 0.1% NaN_3) and after 15 min of capping indicated that those cells with intense staining, i.e., dense SIg, cap more quickly than lightly stained cells.

Our data and the data of others (10) demonstrate that there is a spectrum of densities of SIg on the surface of B cells. If the contractile ap[illegible]atus and membrane fluidity are similar in any population of cells, it is plausible to [illegible]xpect that the density of SIg determines the rate at which any particular cell caps.

Several groups of investigators have shown that as B lymphocytes mature there is a change in the density of surface immunoglobulin (10–12). Sidman and Unanue (12) have reported that early (4 d) murine B cells have a higher density of surface immunoglobulin than adult B cells. Interestingly, fewer neonatal B cells will cap surface immunoglobulin when measured at 45 min, but at 15 min there is an equal number of capped cells in neonatal and adult populations. This suggests that a subset

of early B cells cannot cap. Immaturity of the cytoskeletal system may explain these findings. Scher et al. (10), utilizing flow microfluorimetry, analyzed the shifting density of surface immunoglobulin of individual B cells during ontogeny. This study demonstrates that as B cells mature the density of IgM decreases until 73% of cells have a very low to intermediate density of μ-chain. Analysis of cells stained with a polyvalent immunoglobulin demonstrated that from 3–17 d of age the frequency of cells with very low to intermediate density of surface immunoglobulin increased at the expense of brighter cells. The shift in density of surface immunoglobulin that we have demonstrated may not be a result of a generalized decrease in membrane protein but may be accounted for by the loss of, or decrease in a single isotype.

Surface immunoglobulin capping is dependent on linkage with submembranous contractile elements (13–15). Pharmacologically active anti-tubulins, such as colchicine, are said to have no effect on or slightly enhance surface immunoglobulin capping, suggesting that microtubules play only a small role in this process (16). Colchicine treatment of lymphocytes from young rats increased both the number of caps formed and the rate of capping but did not affect lymphocytes from old rats. Presumably, the microtubular system differs in cells of these two age groups. In young adult rats microtubules appear to constrain ligand-induced modulation because colchicine treatment significantly increases the percentage of capped cells at 20°C and 37°C. The slow capping of lymphocytes obtained from old animals cannot be attributed to microtubule restraint as seen in slow capping murine strains (17), because colchicine did not reverse the slower capping kinetics of old rats.

There also may be changes in membrane fluidity with aging. Preliminary studies indicate a small increase in the microviscosity with aging of both the splenic and lymph node lymphocyte membranes as measured by the anisotropy of the fluorophore 1,6-diphenylhexatriene (B. Woda, J. Yguarabide, and J. Feldman, unpublished data). Measurements obtained with this method have shown that the microviscosity of lymphocyte membranes is dependent upon the molar ratio of cholesterol to phospholipids. An increase in the ratio of cholesterol to phospholipids is associated with an increase in membrane microviscosity and vice versa (18).

If cell-cell interactions and cell-antigen interaction are dependent on recognition by surface molecules, particularly SIg, certain receptor densities may be necessary for recognition and later activation of the cell. A high number of receptors may be necessary for effective antigen binding, and binding of molecules to receptors may have to exceed a finite activation threshold. The ease of tolerance or suppression induction may be dependent on the number of surface receptors.

Summary

The rate of capping and shedding of cross-linked surface immunoglobulins (SIg) was slower in old Lewis rats (> 24 mo) than in young Lewis rats (3–4 mo). Analysis of spleen cell populations with the fluorescence-activated cell sorter indicated that with aging there was a loss of cells with a high density of SIg. Cells with the highest density of SIg capped and shed cross-linked SIg faster than cells with a low density of SIg. The alteration in density of SIg may account for the difference in capping kinetics. Colchicine treatment increased the rate of capping of lymphocytes from young animals, but had no effect on the capping kinetics of lymphocytes from old animals.

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