INTERACTIONS BETWEEN LYMPHOCYTE MEMBRANE
MOLECULES

I. Interaction between B Lymphocyte Surface IgM
and Fc IgG Receptors Requires Ligand Occupancy of Both Receptors

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It is well established that an individual lymphocyte can be affected by more than
one immunologic stimulus. Thus, the net response of a lymphocyte may be determined
by exogenous antigen, by major histocompatibility complex determinants present on
the surface of antigen-presenting cells, and by positive and negative regulatory cells,
and/or their products. Exposure to one or more of these stimuli can have functionally
distinct consequences for the lymphocyte, including cell division, functional differ-
etiation, paralysis, and cell death. Thus, it seems apparent that the lymphocyte must
have a mechanism for distinguishing, integrating, and responding to multiple stimuli.
One possible mechanism for the integration of multiple stimuli by a lymphocyte
would be through interactions between different cell surface molecules.

Two methods have been used to evaluate the existence of such interactions between
lymphocyte surface molecules. In the first method, specific ligand (usually antibody)
is bound to molecule A and the ability to serologically detect molecule B is subse-
quently evaluated. Using this method, interactions have been reported between Ia
antigens and Fc IgG receptors (FcGR)1 (1, 2) and LyM antigens and FcGR (3) on B
lymphocytes, and between Ia antigens and FcGR (4, 5), H-2D b and Lyt 2.2 (6, 7),
H-2K b and Lyt 1.2 (6, 7), H-Y and TL (8), D b and TL (6, 7), and H-Y and D b (8) on
T lymphocytes. The simplest (but not the only) interpretation of these data is that the
interacting molecules are specifically (nonrandomly) located in close proximity to
each other on the lymphocyte surface membrane. Comparisons between fixed and
unfixed cells have shown that in some cases the interaction existed before the
introduction of the ligand, whereas in others it was induced by ligand (7, 8).

In the second method, ligand is bound to molecule A and allowed to redistribute
to one pole of the cell (capping). Cell surface molecule B is then evaluated for evidence
of redistribution paralleling that of molecule A (cocapping). With this method,
interactions have been reported between surface IgM (sIgM) and FcGR (9), the

1 Abbreviations used in this paper: BSA, bovine serum albumin; BSA-PBS, Na phosphate-buffered saline
containing 2% BSA and 0.07% Na azide, pH 7.2; FcGR, Fc IgG receptor; FITC, fluorescein isothiocyanate;
G, goat; H-aggregated IgG, FITC-conjugated heat-aggregated human Cohn fraction II IgG; HBSS-FCS,
Hanks' balanced salt solution without phenol red containing 10% (vol/vol) heat-inactivated fetal calf
serum; LPS, lipopolysaccharide; R, rabbit; S, sheep; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide
gel electrophoresis; sIgD, surface IgD; sIgM, surface IgM; SPA-Sepharose, staphylococcal Protein A-
Sepharose; TMRITC, tetramethylrhodamine isothiocyanate; TNP, trinitrophenyl.
lipopolysaccharide (LPS) receptor and sIgM (10), and surface IgD (sIgD) and the
LPS receptor (10) on B lymphocytes. The simplest (but again, not the only) interpre-
tation of these data is that the two molecules physically interact with each other
directly or indirectly on the cell surface.

The purpose of the present studies was to further characterize the interaction
between two B lymphocyte surface molecules, sIgM and FcGR. The results indicate
that: (a) there is a specific interaction between sIgM and FcGR on the B lymphocyte
surface that requires occupancy of both receptors; and (b) occupancy of the FcGR by
monomeric IgG produces a reversible alteration of this receptor. It is likely that
whenever sIgM is involved in vivo in a B lymphocyte response to an immunologic
stimulus, the FcGR is also involved.

Materials and Methods

Animals and Cells. Adult male mice of strains C57BL/10Sn (B10) and B10.A/SgSn were
purchased from The Jackson Laboratory, Bar Harbor, Maine. Single cell suspensions from
spleen were prepared by density flotation as previously described (1). The cells were resuspended
at 20 x 10^6/ml in Hanks' balanced salt solution without phenol red containing 10% (vol/vol)
heat-inactivated fetal calf serum (HBSS-FCS) for capping studies or in Na-phosphate-buffered
saline containing 2% bovine serum albumin and 0.07% Na azide, pH 7.2, (BSA-PBS) for
noncapping studies.

Fluorescent Reagents

FLUOROCHROME-CONJUGATED F(ab')2 AND F(ab') ANTI-Ig REAGENTS. Rabbits and goats were
immunized with purified myeloma proteins by standard techniques. The antisera obtained
were absorbed using solid-phase immunoadsorbents prepared by covalently coupling purified
myeloma proteins or normal or newborn mouse serum to Sepharose or agarose via cyanogen bromide (11). The IgG fractions of the various antisera were obtained either by affinity
purification using solid-phase immunoadsorbents or DEAE cellulose chromatography. F(ab')2
fragments were prepared by digestion of the IgG with pepsin (12), followed by chromatography
on Sephadex G-150 to isolate the F(ab')2 fraction. Finally, these were absorbed with staphylo-
coccal Protein A-Sepharose (SPA-Sepharose; Pharmacia Fine Chemicals, Div. of Pharmacia Inc.,
Piscataway, N. J.) to remove minor amounts of contaminating intact IgG. Certain of the
F(ab')2 preparations were analyzed for contaminating IgG by sodium dodecyl sulfate-polyacryl-
amide gel electrophoresis (SDS-PAGE). The F(ab')2 preparations were conjugated to fluorescein
isothiocyanate (FITC; 13) or tetramethylrhodamine isothiocyanate (TMRITC) as described
(14). The preparations were evaluated for specificity by the fluorescent staining of spleen cells,
thymocytes, myelomas, and hybridomas of known Ig isotype, as well as Sepharose beads
coupled with various purified myeloma proteins. All reagents were specific in that no inappro-
priate fluorescence was observed. F(ab') fragments were prepared by reduction and alkylation
(15) of the fluorochrome-conjugated F(ab')2 preparations with subsequent isolation of the F(ab')
fraction by Sephadex G-150 chromatography.

(a) Rabbit F(ab')2 and F(ab') Anti-Mouse Mu [R-F(ab')2 and R-F(ab') Anti-Mu]. Fluoro-
chrome-conjugated R-F(ab')2 anti-mu was prepared under contract NCI-CB-53912-31, except
for the absorption with SPA-Sepharose, which was done in our laboratory. The immunogen
was MC774 (lG Mk) and the absorbents were MOPC141 (IgG2bk) and newborn mouse serum.
The concentration used was 250 µg/ml. The preparation was 0.5% contaminated with intact
IgG, which would not bind to SPA-Sepharose. In certain experiments (see Results) the
R-F(ab')2 anti-mu was absorbed with R-anti-TNP-TNP-BSA-Sepharose.

(b) Goat F(ab')2 Anti-Mouse Mu [G-F(ab')2 Anti-Mu]. Absorbed and affinity-purified goat
IgG anti-mouse mu was a kind gift of Dr. Richard Asofsky, National Institutes of Health,
Bethesda, Md. The immunogens were MOPC104 (IgMk) and MC471B (IgMk). The absorbents
were MOPC70A (IgG1k), MOPC173 (IgG2ak), and MOPC195 (IgG2bk). The antibodies were
affinity-purified on TEP183 (IgMk). The concentration used was 15-60 µg/ml. It was 2%
contaminated with intact IgG, which would not bind to SPA-Sepharose.
(c) Rabbit F(\(ab'\)\(_2\))\(_2\) Anti-Mouse Kappa [R-F(\(ab'\)\(_2\))\(_2\) Anti-k]. Fluorochrome-conjugated R-F(\(ab'\)\(_2\))\(_2\) anti-kappa was prepared under contract NCI-CB-53912-31, except for absorption with SPA-Sepharose, which was done in our laboratory. The immunogen was PC5 (IgG2ak) and the absorbent was HOPC1 (IgG2ak). The concentration used was 200 \(\mu\)g/ml.

(d) Rabbit F(\(ab'\)\(_2\))\(_2\) and F(\(ab'\)) Anti-Mouse Delta [R-F(\(ab'\)\(_2\))\(_2\) and F(\(ab'\)) Anti-Delta]. Fluorochrome-conjugated F(\(ab'\)\(_2\)) anti-delta was given and prepared (except for Sephadex G-150 chromatography, which was done in our laboratory) by Dr. Fred D. Finkelman, Uniformed Services University of the Health Sciences, Bethesda, Md. The immunogen was TEPC1017 (IgDk; 16) and the absorbents were normal mouse serum and TEPC183 (IgMk). It was affinity-purified with TEPC1035 (IgDk; 16) and was used at a concentration of 20 \(\mu\)g/ml for F(\(ab'\)\(_2\)) and 150 \(\mu\)g/ml for F(\(ab'\)).

(e) Rabbit F(\(ab'\)\(_2\))\(_2\) Anti-Mouse IgG2 [R-F(\(ab'\)\(_2\))\(_2\) Anti-IgG2]. Fluorochrome-conjugated R-F(\(ab'\)\(_2\))\(_2\) anti-IgG2 was prepared under contract NCI-CB-53912-31 except for absorption with SPA-Sepharose, which was done in our laboratory. The immunogen was PC5 (IgG2ak) and the absorbents were MOPC21 (IgGlk) and MC774 (IgMk). It was used in conjunction with anti-I-A\(^b\) antibody (see below).

Igo Anti-Mouse Ig. Rabbit IgG anti-mouse Ig conjugated with FITC (R-IgG anti-Ig) was purchased from N. L. Cappel Laboratories, Inc., Cochranville, Pa. (lot 7282). It was ultracentrifuged before use (17) and was used at a dilution of 1:8.

Anti-I-A\(^b\) Antibody. IgGlb anti-I-A\(^b\) was purified by affinity chromatography on SPA-Sepharose from tissue culture supernatant fluids of hybridoma 10-2.16 (18; obtained from The Salk Institute, San Diego, Calif.). For the present experiments, it was necessary to prevent this antibody from binding to FcGR via the Fc portion of the molecule. It was found in preliminary experiments that preincubation for 30 min at 4°C with sufficient (>17/1, wt/wt) fluorochrome-conjugated R-F(\(ab'\)\(_2\)) anti-IgG2 would prevent binding of IgG2a (HOPC1) to the FcGR on P388D1 cells (David M. Segal and Howard B. Dickler, unpublished observations). Therefore, equal volumes of 10-2.16 (4.5 \(\mu\)g/ml) and fluorochrome-conjugated RF(\(ab'\)\(_2\)) anti-IgG2 (125 \(\mu\)g/ml) were preincubated for 30 min at 4°C for use in the experiments. The R-F(\(ab'\)\(_2\)) anti-IgG2 also provided labeling for fluorescent detection of I-A\(^b\), and sufficient cross-linking to produce capping (see Results).

Igo Complexes. Two types of complexes were utilized for detection of FcGR on B lymphocytes.

(a) Antigen-Antibody Complexes. The methodology for preparation of the soluble antigen-antibody complexes and their cross-linking and indirect fluorescent detection has been described in detail (19). Briefly, affinity-purified rabbit anti-trinitrophenyl (R-anti-TNP) and TNP-conjugated bovine serum albumin (TNP-BSA) were mixed at fourfold antigen excess to produce soluble complexes that were used at 10 \(\mu\)g/ml (antibody). Further cross-linking and fluorescent detection were obtained with FITC- or TMRITC-labeled affinity-purified sheep (S)-anti-TNP or R-anti-TNP (125-250 \(\mu\)g/ml). The affinity-purified anti-TNP antibodies were the kind gift of Dr. Pierre Henkart, National Institutes of Health, Bethesda, Md.

(b) Heat-aggregated IgG. FITC-conjugated, heat-aggregated human Cohn fraction II IgG (H-aggregated IgG) was prepared as previously described (1) and used at 2 mg/ml.

Monomeric Mouse IgG. Mouse IgG was purchased from Miles Laboratories, Elkhart, Ind. This IgG was chromatographed on Sephadex G-150 and the second half of the 7S peak was isolated, concentrated, and ultracentrifuged immediately before use to remove material \(\geq 10S\) (17). The concentration used was 5 mg/ml. In certain experiments (see Results), the IgG was absorbed with FITC-R-IgG-Sepharose.

Fluorescence. Cells (5 × 10\(^5\) in 25 \(\mu\)l) and reagent (100 \(\mu\)l for R-anti-TNP-TNP-BSA and mouse IgG, 25 \(\mu\)l for all others) were incubated for 30 min at 4°C and then washed thoroughly with 4°C medium. In protocols involving more than one reagent, the reagents were used sequentially in the order noted in Results with thorough washing between each reagent. BSA-PBS (which contained Na azide) was the medium used in protocols not requiring capping, whereas in those that required capping, HBSS-FCS was used until capping was ended with BSA-PBS. Cells were kept at 4°C throughout, except during capping. Capping was induced by resuspending the cells in 1 ml of HBSS-FCS and incubating at 37°C for 15-20 min. Wet mount slides were prepared at the end of each protocol and read under alternate fluorescent and phase
microscopy as previously described (20). A minimum of 200 small lymphocytes per preparation were evaluated. The criterion for fluorescence positivity for each of the molecules evaluated was punctate staining over the entire surface of the cell (except when using monomeric F(ab') antibodies where the pattern was a smooth ring). The fluorescence was considered capped when it was concentrated on <1/2 of the cell surface at one pole of the cell.

Results

Lack of Interaction between slgM and FcGR if Only One of the Receptors Is Ligand-occupied. B lymphocyte slgM and FcGR were evaluated for interactions by binding ligand to one of the two receptors. The cells were subjected to capping or noncapping conditions, followed by immunofluorescent evaluation of the second receptor for detectability and distribution. Fluorochrome-labeled F(ab')2 fragments of rabbit IgG specific for mouse mu [R-F(ab')2 anti-mu] were used for slgM. Soluble antigen-antibody complexes composed of TNP-BSA and rabbit IgG specific for TNP (R-anti-TNP-BSA), followed by fluorochrome-labeled sheep IgG specific for TNP (S-anti-TNP, used for both detection and further cross-linking) were used for FcGR (Table I). The percentage of spleen cells positive for FcGR and the distribution of FcGR on those cells were unaffected by binding of ligand to slgM or ligand-induced redistribution of slgM (parts 5 and 6 vs. part 1). Similarly, the percentage of cells positive for slgM and the distribution of slgM were unaffected by binding of

| Part | Incubation* | sIgM | FeGR |
|------|-------------|------|------|
| 1    | R-anti-TNP-TNP-BSA | S-anti-TNP§ | — | 61.5 | 4 |
| 2    | R-F(ab')2§ anti-mu | — | — | 55.0 | 2 |
| 3    | R-anti-TNP-TNP-BSA | S-anti-TNP | R-F(ab')2 anti-mu | 54.0 | 6 | 61.0 | 5 |
| 4    | R-anti-TNP-TNP-BSA | S-anti-TNP | R-F(ab')2 anti-mu | 56.0 | 5 | 60.0 | 77 |
| 5    | R-F(ab')2 anti-mu | R-anti-TNP-TNP-BSA | S-anti-TNP | 52.0 | 2 | 58.5 | 3 |
| 6    | R-F(ab')2 anti-mu | R-anti-TNP-TNP-BSA | S-anti-TNP | 56.0 | 89 | 58.5 | 4 |

* All incubations were at 4°C for 30 min, and cells were washed thoroughly with iced medium between each incubation. The arrow means that between the indicated incubations the cells were subjected to capping conditions (37°C for 15-20 min in HBSS-FCS). This same medium was used for incubations and washes before capping conditions, whereas BSA-PBS was used for incubations and washes after capping conditions and throughout parts without capping conditions.

‡ Percentage of positive cells with fluorescence visible on ≤1/2 of the cell surface at one pole of the cell.

§ S-anti-TNP was labeled with TMRITC and R-F(ab')2 anti-mu was labeled with FITC.
complexes to FcGR or ligand-induced capping of FcGR (parts 3 and 4 vs. part 2). These results, with one exception, are consistent with the observations of a number of laboratories (reviewed in reference 21) and indicate that the two receptors are independent molecules that are not in close enough proximity on the cell membrane for steric inhibition of detection to occur. However, the result obtained in part 6 differs from that reported by Forni and Pernis (9), who observed that capping of sIgM with F(ab')2 anti-mu resulted in redistribution (cocapping) of FcGR, and from the result of Abbas and Unanue (22), who found cocapping of FcGR after capping with F(ab')2 anti-kappa.

To resolve this discrepancy, sIgM was capped with several different anti-Ig reagents and FcGR were evaluated with two types of complexes (Table II). Capping of sIgM with F(ab')2 fragments of two different anti-mu antibody preparations (one rabbit and one goat) and of an anti-kappa antibody preparation (which would also bind to sIgD) failed to induce redistribution of FcGR (experiment A, parts 1, 2, and 4). Moreover, evaluation of FcGR with different complexes (heat-aggregated IgG) also failed to reveal any cocapping (experiment A, part 3). In contrast, capping with intact rabbit IgG anti-mouse Ig both inhibited FcGR detection and induced FcGR redistribution (experiment A, part 5), presumably due to the Fc portion of the anti-Ig antibodies binding direct to FcGR. In addition, capping with F(ab')2 fragments of anti-mu IgG purified after pepsin digestion by chromatography on Sephadex G-150 but not absorbed with staphylococcal Protein A to remove residual contaminating intact IgG (a procedure not available when the earlier studies [9, 22] were done) also induced some FcGR redistribution (experiment B, part 2). This latter result suggests the possibility that the earlier results (9, 22) were due to small amounts of intact IgG contaminating the F(ab')2 preparations used. Alternatively, it was conceivable that Protein A had leaked from the Protein A-Sepharose, was present in the F(ab')2 anti-

| Table II |

**Effect of Redistributing B Lymphocyte sIgM with Various Anti-Ig Reagents on FcGR**

| Experiment | Part | Incubation* | sIgM‡ | FcGR |
|------------|------|-------------|-------|------|
|            |      | 1 | 2 | 3 | Positive | Capped§ | Positive | Capped |
| A          | 1    | R-F(ab')2 anti-mu | R-anti-TNP anti-mu | S-anti-TNP | 52.0 | 88 | 64.0 | 9 |
|           | 2    | G-F(ab')2 anti-mu | R-anti-TNP anti-mu | S-anti-TNP | 54.0 | 91 | 59.0 | 8 |
|           | 3    | G-F(ab')2 anti-mu | R-anti-TNP anti-mu | S-anti-TNP | 51.5 | 76 | 60.0 | 7 |
|           | 4    | R-F(ab')2 anti-kappa | R-anti-TNP anti-mu | S-anti-TNP | 58.0 | 83 | 22.0 | 91 |
|           | 5    | R-IgG anti-Ig | R-anti-TNP anti-mu | S-anti-TNP | 66.0 | 82 | 62.5 | 4 |
| B          | 1    | R-F(ab')2 anti-mu | R-anti-TNP anti-mu | S-anti-TNP | 65.5 | 86 | 62.0 | 23 |
|           | 2    | R-F(ab')2 anti-mu | R-anti-TNP anti-mu | S-anti-TNP | 64.0 | 91 |

* See footnote to Table I. R-F(ab')2 anti-mu, R-F(ab')2 anti-kappa, R-IgG anti-Ig, H-aggregated IgG, and S-anti-TNP (experiment A, part 2) were labeled with FITC. G-F(ab')2 anti-mu and S-anti-TNP (experiment A, parts 1, 4, and 5, and experiment B) were labeled with TMRITC.

† Anti-kappa and anti-Ig would also bind to sIgD.

§ See footnote to Table I.

ǁ Not absorbed with SPA-Sepharose.
mu, and in some fashion artifactually obviated the sIgM-FcGR interaction. However, this possibility was quite unlikely for several reasons: (a) the SPA-Sepharose was always washed with 6 M guanidine to remove any protein not covalently coupled to the Sepharose before equilibration with the filtration buffer (PBS); (b) no protein (<3 μg/ml) was detected in the PBS, which passed through the SPA-Sepharose; and (c) SDS-PAGE of the R-F(ab')2 anti-mu for contaminants failed to show any protein in the molecular weight range of SPA (42,000) or SPA-IgG complexes even with the gels overloaded (see Materials and Methods). Thus, it was concluded that sIgM and FcGR did not interact if only one of the receptors was occupied by ligand. It was therefore of interest to determine whether the two receptors would interact if they were simultaneously occupied by their respective ligands.

Redistribution of B Lymphocyte sIgM Induces Redistribution of FcGR Independently Occupied by IgG. The experimental approach was to occupy both sIgM [with R-F(ab')2 anti-mu] and FcGR (with antigen-antibody complexes, R-anti-TNP-TNP-BSA) simultaneously, subject the cells to capping conditions, and determine whether capping of sIgM affected the distribution of occupied FcGR (Table III). For this approach to be informative, it is necessary that the antigen-antibody complexes themselves not induce capping of FcGR within the time period analyzed. As previously documented (23), the soluble complexes used in this study induced capping of FcGR on only a minority of the B lymphocytes (experiment A, part 2). This is presumably a reflection of the degree of cross-linking obtained, because if further cross-linking is produced with R-anti-TNP before capping, FcGR capped to approximately the same degree as ligand cross-linked sIgM (experiment A, part 1 vs. 3). When cells were analysed that had both sIgM and FcGR occupied and had been subjected to capping conditions, it was observed that the FcGR had capped nearly as well as when highly cross-linked (experiment A, part 4). Because neither capping of sIgM alone (part 1) nor the soluble

| Experiment Part | Incubation | sIgM | FcGR |
|-----------------|------------|------|------|
| A 1             | R-F(ab')2  | R-anti-TNP- | R-anti-TNP |
|                 | anti-mu    | TNP-BSA |      |
| 2               | R-anti-TNP-| R-anti-TNP |
|                 | TNP-BSA    |       |
| 3               | R-anti-TNP-| R-anti-TNP |
|                 | TNP-BSA    |       |
| 4               | R-F(ab')2  | R-anti-TNP- | R-anti-TNP |
|                 | anti-mu    | TNP-BSA |      |
| B§ 1            | R-F(ab')2  | R-anti-TNP- | R-anti-TNP |
|                 | anti-mu    | TNP-BSA |      |
| 2               | R-anti-TNP-| R-anti-TNP |
|                 | TNP-BSA    |       |
| 3               | R-anti-TNP-| R-anti-TNP |
|                 | TNP-BSA    |       |
| 4               | R-F(ab')2  | R-anti-TNP- | R-anti-TNP |
|                 | anti-mu    | TNP-BSA |      |

* See footnote to Table I. R-F(ab')2 anti-mu was labeled with FITC and R-anti-TNP was labeled with TAMRA.
† See footnote †, Table I.
§ In experiment B, R-F(ab')2 anti-mu was pre-absorbed with R-anti-TNP-TNP-BSA-Sepharose, and R-anti-TNP (in the complex) was pre-absorbed with FITC-R-IgG-Sepharose.
complexes alone (part 2) produced this capping, this result suggested an interaction between slgM and FcGR when both were occupied. One artifact that could also have produced this result was a cross-reaction between the ligands themselves. Although the protocols for preparation of these ligands (see Materials and Methods) should have precluded such cross-reactions, the R-F(\(ab\)'\(2\)) anti-mu was absorbed with insolubilized complexes, the R-anti-TNP used in the complexes was absorbed with insolubilized rabbit IgG conjugated with FITC, and the experiment was repeated (experiment B). The results were identical, making it very unlikely that ligand cross-reactions were the explanation. In a series of 12 independent experiments, 86% of the B lymphocytes whose slgM was redistributed into a cap also had capped FcGR, provided the latter were occupied at the time by complexes. The same result was also obtained with spleen cells from other strains including C3H/HeJ, A/J, and CBA/N (data not shown). It was concluded that slgM and FcGR interact if both are ligand-occupied.

Because FcGR are constantly exposed in vivo to monomeric IgG, it was of interest to determine whether FcGR occupied by monomeric IgG would also interact with slgM. The experimental approach was similar to that used for complexes with two modifications necessitated by the low binding avidity of monomeric IgG to B lymphocyte FcGR. First, the monomeric IgG was continuously present during the capping period, and second, the distribution of FcGR was subsequently detected by labeling with complexes and fluorochrome-labeled R-anti-TNP (Table IV). When cells labeled with R-F(\(ab\)'\(2\)) anti-mu were subjected to capping conditions in the continuous presence of monomeric IgG and then analyzed for the distribution of FcGR, it was observed that approximately half of the B lymphocytes had all their

| Table IV |
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| Redistribution of B Lymphocyte slgM Induces Redistribution of FcGR Occupied by Monomeric IgG |

| Part | Incubation* | FcGR |
|---|---|---|
|   | 1 | 2 | 3 | 4 | Positive Capped‡ |
|   | % | | | | |
| 1 | R-F(\(ab\)'\(2\)) anti-mu | R-anti-TNP-TNP-BSA | R-anti-TNP | — | 54.0 | 11 |
| 2 | R-F(\(ab\)'\(2\)) anti-mu | R-anti-TNP-TNP-BSA | R-anti-TNP | — | 54.0 | 81 |
| 3 | R-F(\(ab\)'\(2\)) anti-mu | R-anti-TNP-TNP-BSA | R-anti-TNP-TNP-BSA | R-anti-TNP | 60.0 | 47 |
| 4 | Mouse IgG§ anti-mu | R-anti-TNP-TNP-BSA | R-anti-TNP-TNP-BSA | R-anti-TNP | 56.0 | 46 |
| 5 | Mouse IgG§ anti-mu | R-anti-TNP-TNP-BSA | R-anti-TNP-TNP-BSA | R-anti-TNP | 54.0 | 15 |
| 6 | Mouse IgG§ anti-mu | R-anti-TNP-TNP-BSA | R-anti-TNP-TNP-BSA | R-anti-TNP | 55.0 | 11 |

* See footnote to Table I. R-F(\(ab\)'\(2\)) anti-mu was labeled with FITC and R-anti-TNP was labeled with TMRITC.
‡ See footnote to Table I; slgM was 53.0% positive and 75% capped.
§ Mouse IgG was pre-absorbed with FITC-R-IgG-Sepharose, chromatographed on Sephadex G-150, and ultracentrifuged immediately before use (see Materials and Methods). In parts 4 and 6, the IgG was present during capping conditions at a final concentration of 0.5 mg/ml. In part 5, the IgG was washed away before capping.
FcGR redistributed into a cap (part 4). Neither capping of sIgM alone (part 1) nor monomeric IgG alone (part 6) induced this redistribution. The fact that FcGR were redistributed into a cap on only half the B lymphocytes is more apparent than real. First, because of the subjective nature of the assay, only cells in which all FcGR are at one pole of the cell are counted as capped. In fact, although many of the B lymphocytes in part 4 had most of their FcGR in a cap, some were elsewhere on the cell surface and so were counted as noncapped. Second, previous studies have shown that after capping of FcGR directly by highly cross-linked complexes, reexposure to complexes shows some FcGR outside the cap (24, 25). It is thought that this is due to rapid reexpression of FcGR. A similar phenomenon was apparent in the present experiments. Thus, FcGR occupied by soluble complexes during capping of sIgM were found capped on 81% of the positive cells (part 2). However, if the cells were reincubated with complexes after capping, all the FcGR were in the cap on only 47% of the cells (part 3), a result equivalent to that obtained with monomeric IgG. It was concluded that occupancy of FcGR by monomeric IgG is sufficient to lead to interaction with sIgM on the majority of B lymphocytes, although for technical reasons this can only be directly demonstrated on half of the B cells.

Several other points are worth noting. Cross-reactions between the ligands were excluded by absorptions (see Materials and Methods and Table IV, footnote §). Also, every effort was made to insure that the IgG was monomeric i.e., Sephadex G-150 chromatography was planned so that the material obtained (second half of the 7S peak) was not stored before use and was ultracentrifuged to remove dimers or larger immediately before use. In addition, fresh normal mouse serum produced a similar result (data not shown). The concentration of IgG required (0.5 mg/ml) is considerably less than is present in serum and titration experiments indicated that as little as 0.01 mg/ml produced some effect (data not shown). Finally, the effect of the monomeric IgG on FcGR is completely reversible because if it is washed away immediately before capping, redistribution of FcGR does not occur (part 5).

Lack of Interaction between Immune Complex-occupied FcGR and sIgM Occupied by Monomeric Anti-Mu. To further characterize the interaction between sIgM and FcGR, we asked whether the capping of FcGR by highly cross-linked complexes would induce redistribution of sIgM occupied by monomeric anti-mu (Table V). Lymphocytes were

| Part | Incubation* | slgM | FcGR |
|------|-------------|------|------|
|      |             |      |      |
| 1    | R-F(ab') anti-mu | 55.0 | 4    |
| 2    | R-F(ab') anti-mu | 54.5 | 85   |
| 3    | R-anti-TNP-BSA | 57.0 | 89   |
| 4    | R-anti-TNP-BSA | 54.0 | 92   |

* See footnote to Table I. R-F(ab') anti-mu and R-F(ab') anti-mu were labeled with FITC and R-anti-TNP was labeled with TRITC.
‡ See footnote to Table I.
allowed to bind soluble R-anti-TNP-TNP-BSA complexes, which were then further
cross-linked by additional R-anti-TNP. The sIgM was then labeled with R-F(ab')
anti-mu and the cells were subjected to capping conditions (part 4). Although the
FcGR were capped on 92% of the positive cells, no redistribution of sIgM occurred.
The control (part 2) indicated that sIgM would cap normally if cross-linked by
divalent F(ab')2 anti-mu (from which the monomer was prepared by reduction and
alkylation). This result conflicts with that reported by Unanue and Abbas (26).
However, it was later found that the latter result was due to a cross-reaction between
ligands (E. R. Unanue, personal communication). Thus, an interaction between sIgM
and FcGR could not be demonstrated if sIgM was occupied by monomeric ligand.
The simplest explanation would be that sIgM must be cross-linked in order to interact
with FcGR. Because cross-linked sIgM caps very rapidly, this possibility could not be
directly investigated with the present experimental approach.

Specificity of the Interaction between sIgM and FcGR. It was of interest to determine
whether interactions would occur between any two ligand-occupied cell surface
molecules on B lymphocytes, as opposed to the possibility that the sIgM-FcGR
interaction was unique. Ligand-induced capping of sIgM failed to elicit redistribution
of sIgD occupied by monomeric anti-delta (Table VI, part 5). Moreover, no evidence
of any interaction between sIgM and sIgD was obtained. Thus, binding of ligand to
one of these molecules did not inhibit detection of the second molecule, nor did
capping of one of the molecules induce redistribution of the other, irrespective of
whether the second was ligand-occupied (Table VI). Capping of sIgM also did not
induce significant redistribution of antibody-bound I-A antigens (Table VII, part 5
vs. part 2). It is worth noting that the antibody for I-A was at least divalent. This was
possible because even when highly cross-linked, this molecule capped slowly. It would,
however, cap on the majority of B cells, given sufficient time (part 3). Thus, capping
of sIgM induced redistribution of ligand-occupied FcGR but not ligand-occupied
sIgD or I-A antigens.

Experiments were also carried out to determine whether capping of another B
lymphocyte surface molecule (sIgD) would induce redistribution of ligand-occupied
FcGR. It was found that capping of sIgD induced redistribution of ligand-occupied
FcGR but the interaction was distinct from that with sIgM in that (a) it occurred

| Part | Incubation* | sIgM | sIgD |
|------|-------------|------|------|
| 1    | G-F(ab')2 anti-mu | R-F(ab')2 anti-delta | 50.0 | 47.0 |
| 2    | R-F(ab')2 anti-delta | G-F(ab')2 anti-mu | 48.0 | 49.5 |
| 3    | G-F(ab')2 anti-mu ↔ R-F(ab')2 anti-delta | 50.0 | 49.4 |
| 4    | R-F(ab')2 anti-delta ↔ G-F(ab')2 anti-mu | 49.0 | 51.0 |
| 5    | G-F(ab')3 anti-mu | R-F(ab') anti-delta ↔ | 51.0 | 49.0 |
| 6    | R-F(ab')3 anti-delta | R-F(ab') anti-mu ↔ | 51.0 | 50.0 |

* See footnote to Table I. G-F(ab')2 anti-mu and R-F(ab')2 anti-delta (part 6) were labeled with TMRITC.
R-F(ab')2 anti-delta, R-F(ab') anti-delta, and R-F(ab') anti-mu were labeled with FITC.
‡ See footnote to Table I.
TABLE VII

Redistribution of B Lymphocyte slgM Does Not Induce Redistribution of Antibody-bound I-A Antigens

| Part | Incubation* | slgM | I-A³ |
|------|-------------|------|------|
|      |             | 1    | 2    | Positive | Capped | Positive | Capped |
| 1    | Anti-I-A    | —    | —    | 54.0     | 0      | —        | —      |
| 2    | Anti-I-A    | ↔    | —    | 54.0     | 7      | —        | —      |
| 3    | Anti-I-A§   | ↔    | —    | 55.0     | 69     | —        | —      |
| 4    | G-F(ab')2 anti-mu ↔ Anti-I-A | 51.0 | 82 | 54.0 | 0 |
| 5    | G-F(ab')2 anti-mu Anti-I-A ↔ | 52.0 | 78 | 53.0 | 12 |

* See footnote to Table I. Spleen cells were B10.A. Anti-I-A = IgG2 anti-I-A³ (10-2.16) preincubated with 27 times (wt/wt) of R-F(ab')2 anti-IgG labeled with FITC. The latter reagent prevents the anti-Ia antibody from binding to FcGR (see Materials and Methods) in addition to producing cross-linking and labeling. G-F(ab')2 anti-mu was labeled with TMRITC.

§ See footnote to Table I.

Discussion

The experimental results presented indicate an interaction between two independent B lymphocyte surface membrane receptors, the IgM receptor for antigen, and the receptor specific for the Fc portion of IgG. This interaction required simultaneous occupancy of both receptors by independent ligands. Monomeric ligand (IgG) bound to FcGR induced interaction, whereas divalent ligand was required for slgM. The interaction was specific in that it did not occur between other pairs of ligand-occupied B cell surface molecules, e.g., slgM-slgD and slgM-I-A antigen. Although the interaction was demonstrated via the phenomenon of cocapping, the results in no way suggest, nor do we believe, that capping itself is important in B cell differentiation or function. Two major questions raised by these results are (a) what is the nature of the slgM-FcGR interaction? and (b) what is its physiologic significance?

It is likely that slgM and FcGR interact physically, directly or indirectly. The strongest evidence for this conclusion comes from the observation that FcGR occupied by monomeric IgG can be induced to cap by simultaneously capping slgM. This is, to our knowledge, the first example of a membrane molecule capping when occupied by ligand that is itself unable to produce capping. Because the capping process depends on cross-linking in a variety of systems (reviewed in reference 27), it seems likely that the ligand-occupied slgM is inducing cross-linking of the IgG-occupied FcGR. This could take place via a direct slgM-FcGR binding or could be mediated by an intermediate molecule. Although the present experiments do not exclude an intermediate, we favor a direct binding between slgM and FcGR because this would be more economical and because of the apparent specificity of the interaction. The binding of ligand-occupied slgM to ligand-occupied FcGR would presumably be noncovalent because the interaction induced by monomeric IgG was completely reversible. The requirement of ligand occupancy for both slgM and FcGR suggests...
that the binding of ligands alters these receptors, leading to the exposure of binding sites. Such alterations could be conformational or due to exposure of sites previously not available, e.g., hidden in the membrane. At present, there is no evidence to favor either of these possibilities. Our view of the interaction between sIgM and FcGR is presented schematically in Fig. 1.

In view of the fact that the sIgM-FcGR interaction occurs in the presence of monomeric IgG at concentrations present in vivo, it appears likely that whenever sIgM is involved in a B lymphocyte response via interaction with antigen, the FcGR would also be involved. Based on evidence that the ligand anti-\(\mu\) antibody can induce proliferation of B lymphocytes (28, 29), i.e., signal the B cell, this suggests that the sIgM-FcGR interaction is important in signaling the B lymphocyte. We would like to propose that this interaction can provide either positive or negative signals to the B cell, depending on the nature of the ligand occupying the FcGR.

In terms of a negative signal to the B lymphocyte, there is a substantial amount of evidence implicating the FcGR. It has been known for several years that antigen-antibody complexes can inhibit the antibody response in an antigen-specific manner, and that this inhibition requires an intact Fc portion of the antibody (30). However, because such antibody responses require several collaborating cell types, many of which bear FcGR, it has been difficult to demonstrate that this effect was mediated by the FcGR of the B lymphocyte (31). In contrast, other model systems have more
directly implicated the FcGR of B lymphocytes in negative signaling. Both LPS and anti-mu antibody-induced proliferation of B lymphocytes can be inhibited by antibodies bound to the B cell surface and this inhibition requires an intact Fc portion of the antibody (32, 33). slgM has also been implicated in negative signaling. Under circumstances in which slgM is the predominant Ig isotype (neonatal B cells or B cells from the CBA/N strain) or the exclusive isotype (due to removal of slgD by enzymes or modulation with anti-delta antibodies) on the majority of B lymphocytes, then tolerance induction (a type of negative signal) is greatly facilitated (34–36). Thus, the available evidence is consistent with the possibility that the slgM-FcGR interaction would provide a negative signal to the B lymphocyte when the FcGR is occupied by antibody.

In terms of positive signaling, two groups of workers have proposed (37, 38), on the basis of indirect evidence, that the FcGR is the site of binding for T lymphocyte helper factor. Moreover, it has recently been demonstrated that B lymphocytes in the presence of F(ab')2 anti-mu and T cell helper factor will both proliferate and differentiate into antibody-forming cells (39). Thus, it seems possible that the slgM-FcGR interaction could provide a positive signal to the B lymphocyte if the FcGR was occupied by T cell helper factor.

The proposal that the interaction of slgM and FcGR can provide both positive and negative signals to the B cell depending on the nature of the ligand bound to the FcGR requires two further elements. First, how does the FcGR know which ligand to bind? The simplest mechanism would be competition for the receptor based on relative affinities of the ligands, and the order (a) monomeric IgG, then (b) T cell helper factor, then (c) complexed IgG would seem the most reasonable. Second, how does the cell know which ligand is bound to the FcGR? Two mechanisms could accomplish this. Either IgG and T cell helper factor bind to different sites on the FcGR (in which case the competition mentioned above would be steric), or the cell possesses a recognition mechanism that distinguishes FcGR-IgG from FcGR-T cell helper factor. No evidence is available to distinguish these possibilities.

The hypotheses put forward above appear to be testable with available experimental techniques. It will also be of interest to examine other cell surface molecules for interactions and determine whether such interactions have general importance in cell signaling.

Summary

The independent B lymphocyte surface membrane receptors IgM and Fc IgG receptors were evaluated for interactions using immunofluorescence. Ligand [F(ab')2 anti-mu]-induced capping of surface IgM resulted in capping of Fc IgG receptors only if the latter were occupied during the capping process by: (a) soluble antigen-antibody complexes that themselves provided insufficient cross-linking to result in capping; or (b) monomeric IgG at physiologic concentrations (or less) either purified or as normal serum. Ligand-induced capping of Fc IgG receptors did not result in capping of surface IgM occupied by monomeric F(ab') anti-mu. Control experiments showed that ligand binding to or capping of only one of these two receptors has no effect on the other, and that there were no cross-reactions. The interaction appears specific in that ligand-induced capping of surface IgM did not induce capping of ligand-occupied surface IgD or I-A antigens. Thus, there appears to be a specific
interaction between ligand-bound surface IgM and ligand-bound Fc IgG receptors on
the B lymphocyte surface. The results also indicate that binding of monomeric IgG
produces a reversible alteration in the Fc IgG receptor leading to association with
ligand-bound surface IgM. Because Fc IgG receptors are continuously exposed to
monomeric IgG in vivo, these results suggest that whenever surface IgM is involved
in a B lymphocyte response to an immunologic stimulus, the Fc IgG receptor is also
involved.

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