POLYCLONAL ACTIVATION OF MURINE LYMPHOCYTES BY ANTIBODY TO CELL SURFACE IGD

F. D. Finkelman, J. J. Mond, I. Scher, S. Kessler and E. S. Metcalf

J. Vorosmarti, CAPT, MC, USN
Commanding Officer
Naval Medical Research Institute

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Naval Medical Research Institute
Bethesda, Maryland 20814

Naval Medical Research & Development Command
Bethesda, Maryland 20814

Bureau of Medicine & Surgery
Department of the Navy
Washington, D.C. 20372

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Polyclonal B-Cell Activation
Anti-IgD
Mice
Lymphocytes

see reverse side
The availability of hybridoma produced anti-δ antibodies and murine IgD secreting plasmacytomas made it possible to examine in vivo activation of murine lymphocytes by anti-δ antibodies using anti-δ concentrations similar to those achieved in in vitro activation. Within 24 hrs after i.v. injection of 800 μg of affinity purified goat anti-mouse δ (GaMδ) antibody splenic B cells increased their quantities of cell surface (s) Ia antigen, their size, and their rate of DNA synthesis. All three processes are T-independent.

The data suggest that in vivo B cell activation by soluble anti-δ proceeds through two phases. The first, includes increases in sIa density, cell size, and rate of DNA synthesis and appears to be T-independent. The second phase, includes a further increase in the rate of DNA synthesis, acquisition of sIgG, and Ig secretion, appears to be T-dependent and, to a large extent, carrier dependent, which suggests that T cell help specific for goat Ig may be polyclonally focused onto B cells by GaMδ antibody.
MULTINUCLEAR ACTIVATION OF MURINE LYMPHOCYTES BY ANTIBODY TO CELL SURFACE IGG

FRED D. FISCHERMAN† JAMES J. WOOD† IMIN SCHER‡ STEVEN W. KESSLER†

†Department of Medicine, Uniformed Services University of the Health Sciences, ‡Department of Immunology, Naval Medical Research Institute, ‡‡Department of Microbiology, Uniformed Services University of the Health Sciences, Bethesda, Md. 20014

INTRODUCTION

The discovery that IgD is present on most human B lymphocytes1-3 was rapidly followed by its identification as a major B lymphocyte surface isotype in several other mammalian species,4-7 as well as by attempts to ascertain its function. Many of these efforts have used antibodies prepared against IgD as functional probes with the thought that anti-IgD would reproduce polyclonally the changes induced in B cells when their surface IgD bound antigen. In vitro experiments have shown anti-IgD antibodies to block antigen specific responses to a variable extent4-10 and to induce B lymphocytes to proliferate.11-14 Such cells, when further stimulated by macrophage or T cell derived factors, differentiate into antibody secreting cells.14 While the culture conditions and physical forms of anti-IgD antibodies required to achieve B cell activation have varied, it is generally agreed that high anti-IgD concentrations (i.e., 50-100 µg/ml) are required to maximize B cell proliferation.11-13

In vivo experiments performed in monkeys,15-17 rats,18 and mice10,19 have generally indicated that anti-IgD can increase the humoral immune response to high doses of simultaneously injected antigen but does not induce polyclonal B cell activation. The recent availability of hybridoma produced anti-IgD antibodies20,21 and murine IgD secreting plasmacytomas22 has made it possible for us to re-examine in vivo activation of murine lymphocytes by anti-IgD antibodies using anti-IgD concentrations similar to those achieved in in vitro activation experiments. We find that within 48 hrs after i.v. injection of 400 µg of affinity purified rat anti-mouse IgD antibody splenic B cells have increased their quantities of cell surface IgD antigen, their size, and their rate of DNA synthesis. All three processes are T-independent. Six-seven days after anti-IgD injection a second phase of activation is observed which is T-dependent. Splenic T cells as well as B cells are found to be proliferating, a 3-4 fold increase in the number of spleen cells is seen, large numbers of B cells with IgD are found, and a polyclonal increase in the secretion of both
IgM and IgG is observed. This state of polyclonal immune activation persists as long as anti-δ antibody remains in circulation; once injected anti-δ is catabolized the immune system reverts to its initial level within one week.

GENERAL PROCEDURES

Following i.v. injection of 800 μg of GaMδ or control antibody BALB/c mice were sacrificed after varying periods of time. Portions of spleen and lymph nodes were formalin-fixed, sectioned, stained with hematoxylin and eosin and examined microscopically. Single cell suspensions prepared from these organs were analyzed for volume with a Coulter Channelizer and for DNA content by staining with an ethanolic solution of Mithramycin and determining cellular fluorescence intensity with a Becton-Dickinson FACS II fluorescence activated cell sorter. Cells were analyzed for surface markers by staining with an FITC-labeled monoclonal antibody specific for Thy 1.2 (Clone 30H-12) or with FITC-labeled affinity purified F(ab')2 fragments of rabbit antibodies specific for mouse δ, μ, or γ chains or keyhole limpet hemocyanin (control) or with A.TH anti-A.TL (anti-Ia) or normal mouse serum (control) followed by FITC-labeled rabbit anti-mouse γ, after which the percentage of cells positive for each surface marker as well as the median fluorescence intensity of specifically stained cells was determined with a FACS II. Surface Ig was also studied by radioautographic analysis of an SDS-PAGE electropherogram of an immunoprecipitated NP-40 extract of 125I surface labeled spleen and lymph node cells from anti-δ injected and control mice. The percentages of cells with intracytoplasmic IgM or IgG were determined by staining methanol fixed cytocentrifuge preparations of these cells with the appropriate FITC-labeled antibodies and examining the stained preparations by fluorescence microscopy. In some experiments the percentages of cells secreting IgM or IgG were determined by the Protein A reverse plaque assay and the percentages of cells secreting IgM anti-TNP were determined by a modified Jerne plaque assay. In some experiments mice were injected with 3H-thymidine 18-24 hrs prior to sacrifice and the amount of 3H incorporated per 2 x 10^6 cells was analyzed by scintillation spectroscopy.

RESULTS

I. Early Events. Twenty-four hrs after injection of GaMδ the ratio of white pulp: red pulp in spleen is greatly increased and the small B lymphocytes of the mantle layers of spleen and lymph node follicles appeared to have differentiated into cells with increased amounts of pale staining cytoplasm.
and large pale nuclei with prominent nucleoli. Surface IgD was almost totally
retrieved from splenic B lymphocytes, which retained most of their size. No
significant changes were seen in percentages of sigI or sia- cells, although
the fluorescence intensity of sia staining was markedly increased (Table 1).

TABLE I

EFFECTS OF GAMS ON SPLEEN CELL SURFACE MARKERS

<table>
<thead>
<tr>
<th>Day</th>
<th>Antigen Injected</th>
<th>Percent Positive (Median Fluorescence Intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>SL G IgG</td>
<td>55.3</td>
</tr>
<tr>
<td></td>
<td>GAMS</td>
<td>&lt;2</td>
</tr>
<tr>
<td>3</td>
<td>SL G IgG</td>
<td>50.0</td>
</tr>
<tr>
<td></td>
<td>GAMS</td>
<td>9.3</td>
</tr>
<tr>
<td>7</td>
<td>SL G IgG</td>
<td>51.8</td>
</tr>
<tr>
<td></td>
<td>GAMS</td>
<td>9.1</td>
</tr>
<tr>
<td>14</td>
<td>SL G IgG</td>
<td>51.7</td>
</tr>
<tr>
<td></td>
<td>GAMS</td>
<td>17.7</td>
</tr>
<tr>
<td>26</td>
<td>SL G IgG</td>
<td>47.5</td>
</tr>
<tr>
<td></td>
<td>GAMS</td>
<td>21.1</td>
</tr>
</tbody>
</table>

* Days after injection of 800 µg of normal goat IgG or GAMS.

Pools of spleen cells from 3 mice were stained directly for IgD, IgM, IgG,
or Thy 1, or indirectly for Ia (anti-ATL + FITC-Rabbit) and analyzed with
a fluorescence activated cell sorter. Median fluorescence intensity is a
measure of percent of stained percent of cells expressing that marker.

** N.D. = Not done

†分析 staining of the 2% percentage of cell staining directly

‡ Cells in this study were injected with 3 µg of normal goat IgG or IgM on
days 4, 11, 26, and 26.

These changes were associated with marked increases in spleen cell size
(Table 2) and DNA synthesis (Table 3). Twenty-four hours after GAMS injection
the percentage of spleen cells with greater than 2C DNA was more than 2 fold
increased as compared to control values (Table 3). Three days after GAMS
injection spleen cells from anti-I treated mice showed 4 times more in vivo
3H-thymidine incorporation on a per cell basis than spleen cells from control
TABLE 2
EFFECT OF GaM6 ON SPLEEN CELL SIZE

<table>
<thead>
<tr>
<th>Day</th>
<th>Antibody Injected</th>
<th>Percent of Spleen Cells with a Volume of (113-248 \mu^3)</th>
<th>253-423 (\mu^3)</th>
<th>429-626 (\mu^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NI G IgG</td>
<td>74 ± 1</td>
<td>21 ± 1</td>
<td>6 ± 3</td>
</tr>
<tr>
<td></td>
<td>GaM6</td>
<td>51 ± 1</td>
<td>34 ± 1</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>3</td>
<td>NI G IgG</td>
<td>82 ± 2</td>
<td>15 ± 1</td>
<td>3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>GaM6</td>
<td>37 ± 3</td>
<td>44 ± 1</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>7</td>
<td>NI G IgG</td>
<td>85 ± 2</td>
<td>13 ± 1</td>
<td>2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>GaM6</td>
<td>29 ± 2</td>
<td>46 ± 1</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>14</td>
<td>NI G IgG</td>
<td>84 ± 1</td>
<td>14 ± 0.9</td>
<td>2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>GaM6</td>
<td>79 ± 4</td>
<td>18 ± 3</td>
<td>3 ± 0.6</td>
</tr>
<tr>
<td>13*</td>
<td>NI G IgG</td>
<td>76 ± 7</td>
<td>20 ± 5</td>
<td>5 ± 2</td>
</tr>
<tr>
<td></td>
<td>GaM6</td>
<td>35 ± 3</td>
<td>43 ± 1</td>
<td>22 ± 4</td>
</tr>
</tbody>
</table>

* Days after injection of 800 \(\mu g\) of normal goat IgG or GaM6.
  Mice were injected with 800 \(\mu g\) of normal goat IgG or GaM6 on days, 0, 1, 5, 7, 10, 11, and 12.
  † Arithmetic mean ± standard deviation of spleen cells from 3 mice.

Mice. Sorting of these cells into \(\text{sIa}^+\) and \(\text{sIa}^-\) fractions prior to Coulter analysis and scintillation spectroscopy demonstrated that only the \(\text{sIa}^+\) cells from GaM6 treated mice showed increased size and \(^{3}H\)-thymidine incorporation. These events appeared to be T-independent, since congenitally athymic (nu/nu) mice and mice tolerized to goat IgG by i.v. injection of 2 mg of ultracentrifuged goat IgG 2 weeks prior to GaM6 injection behaved similarly to normal non-tolerized GaM6 treated mice during the first 3 days after anti-\(\delta\) injection.

Anti-\(\delta\) induced increases in \(\text{sIa}\) density, B cell size, and B cell DNA synthesis, are, to some extent, independent events in B cell activation. Injection of 100 \(\mu g\) of GaM6 induced a substantial increase in \(\text{sIa}\) density but not B cell size. B cells from mice with the CBA/N X-linked immune defect \(^{23}\) increased in size in response to GaM6 both in vivo and in vitro (A. DeFranco and W. E. Paul, personal communication) but did not show increased DNA synthesis.

II. Latent Events. Six - seven days after GaM6 injection the immune system demonstrated further evidence of polyclonal activation. The frequency of \(\text{sIgG}^+\)
TABLE 3

EFFECTS OF GaM6 ON SPLEEN CELL NUMBER AND DNA SYNTHESIS

<table>
<thead>
<tr>
<th>Day</th>
<th>Antibody Injected</th>
<th>Cell Number x 10^6</th>
<th>(^{3}H)-Thy CPM/2 x 10^6 Cells†</th>
<th>Percent &gt;2C DNA**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NL G IgG</td>
<td>101 (1.13)***</td>
<td>787 (1.10)***</td>
<td>4.7 (1.60)***</td>
</tr>
<tr>
<td></td>
<td>GaM6</td>
<td>86 (1.10)</td>
<td>714 (1.30)</td>
<td>10.4 (1.26)</td>
</tr>
<tr>
<td>3</td>
<td>NL G IgG</td>
<td>78.6 (1.06)</td>
<td>548 (1.17)</td>
<td>5.5 (1.23)</td>
</tr>
<tr>
<td></td>
<td>GaM6</td>
<td>119 (1.10)</td>
<td>2,257 (1.18)</td>
<td>12.2 (1.08)</td>
</tr>
<tr>
<td>7</td>
<td>NL G IgG</td>
<td>75.9 (1.17)</td>
<td>551 (1.17)</td>
<td>5.5 (1.11)</td>
</tr>
<tr>
<td></td>
<td>GaM6</td>
<td>352 (1.22)</td>
<td>1,866 (1.85)</td>
<td>12.1 (1.34)</td>
</tr>
<tr>
<td>14</td>
<td>NL G IgG</td>
<td>111 (1.08)</td>
<td>N.D.</td>
<td>3.4 (1.08)</td>
</tr>
<tr>
<td></td>
<td>GaM6</td>
<td>115 (1.15)</td>
<td>N.D.</td>
<td>4.6 (1.17)</td>
</tr>
<tr>
<td>13††</td>
<td>NL G IgG</td>
<td>169 (1.53)</td>
<td>N.D.</td>
<td>6.4 (1.70)</td>
</tr>
<tr>
<td></td>
<td>GaM6</td>
<td>263 (1.63)</td>
<td>N.D.</td>
<td>22.6 (1.21)</td>
</tr>
</tbody>
</table>

*Days after injection of 800 μg of normal goat IgG or GaM6.
**Determined by fluorescence intensity after staining with mithramycin.
††Geometric mean and standard deviation of spleen cells from 3 mice.
†CPM of \(^{3}H\)-Thymidine/2 x 10^6 Nucleated Spleen Cells 18 hrs after i.v. injection of 100 μCI of \(^{3}H\)-Thymidine.
†††Mice were injected with 800 μg of normal goat IgG or GaM6 on days 0, 1, 5, 7, 10, 11, and 12.
N.D. = Not Done.

Spleen cells increased to 4-8 times the normal level (Table 1). Some of this increase may have been due to formation of GaM6 - mouse anti-goat Ig immune complexes that bound to Fc receptors. However, the appearance of a large percentage of Thy 1-8IgM cells at this time favors the possibility that increased numbers of B cells with intrinsic IgG were appearing. In addition, SDS-PAGE analysis of reduced \(^{125}I\)-labeled Ig from these cells indicated the presence of a major band with mobility slightly faster than that of \(\delta\) chain and slower than that of the heavy chain of serum IgG\(_{2a}\). This major band, which is found only in trace amounts on lymphoid cells from control mice, has a mobility similar to that of previously described surface Y\(_{2a}\) from murine tumor cell lines.24,25

Spleen cells from GaM6 treated mice 7 days after injection continued to show increased size and DNA synthesis. However, analysis of FACS purified...
TABLE 4
EFFECT OF GaM5 ON GENERATION OF SPLEEN CELLS THAT EXPRESS CYTOPLASMIC Ig

<table>
<thead>
<tr>
<th>Day *</th>
<th>Antibody Injected</th>
<th>Percent of Cells Expressing Cytoplasmic IgM</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>NL G IgG</td>
<td>0.9 (1.45)</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td></td>
<td>GaM5</td>
<td>2.2 (1.53)</td>
<td>0.4 (2.03)</td>
</tr>
<tr>
<td>7</td>
<td>NL G IgG</td>
<td>0.4 (1.09)</td>
<td>0.5 (2.14)</td>
</tr>
<tr>
<td></td>
<td>GaM5</td>
<td>2.5 (1.67)</td>
<td>15.7 (1.28)</td>
</tr>
<tr>
<td>14</td>
<td>NL G IgG</td>
<td>0.2 (1.46)</td>
<td>0.3 (1.88)</td>
</tr>
<tr>
<td></td>
<td>GaM5</td>
<td>0.7 (1.29)</td>
<td>1.4 (1.60)</td>
</tr>
<tr>
<td>13**</td>
<td>NL G IgG</td>
<td>1.5 (1.14)</td>
<td>3.1 (1.54)</td>
</tr>
<tr>
<td></td>
<td>GaM5</td>
<td>6.9 (1.39)</td>
<td>33.0 (1.26)</td>
</tr>
</tbody>
</table>

*Days after injection of 800 μg of normal goat IgG or GaM5.
Mice were injected with 800 μg of normal goat IgG or GaM5 on days 0, 1, 5, 7, 10, 11, and 12.
†Geometric mean and standard deviation of spleen cells from 3 mice.

Mice injected with 800 μg of normal goat IgG or GaM5 showed an increase in size and rate of DNA synthesis. These findings are consistent with the observation that absolute numbers of both B and T cells in spleen were now substantially increased (calculated from Tables 1 and 3).

Increases in synthesis of IgG and IgM by spleen cells were also noted 7 days after GaM5 injection. This was evident from the percentages of cells that contained intracytoplasmic IgM and IgG (Table 4) as well as from approximately 10 fold increases in frequencies of Ig secreting cells (determined by the protein A reverse plaque technique) total Ig secretion (determined by in vitro incorporation of 3H-leucine into IgM and IgG), and cells secreting IgM anti-Thy1 antibodies. This last finding is of particular importance as it indicates that the increased Ig synthesis stimulated by GaM5 represents a polyclonal antibody response rather than simply an enhanced anti-goat Ig response. In congenitally athymic mice no increase in the frequency of sIgG+sThy1 lymphocytes, rate of lymphocyte DNA synthesis, spleen cell number, or Ig synthesis were observed 7 days after GaM5 injection. Similarly, mice "tolerized" with 2 mg of ultracentri-
fused goat IgG showed much less evidence of polyclonal activation 7 days after GaM6 injection than did non- tolerized mice.

III. Reversal of Activation. Ten days after GaM6 injection circulating anti-δ was no longer detectable and considerable numbers of sIgD+ cells were seen. Decreases in the size of splenic lymphocytes and a reduction in the number and frequency of sIgD+ cells were also observed. By 14 days after anti-δ injection these parameters (Table 1-3), as well as the frequency of cells with intracytoplasmic Ig (Table 4) and the histologic appearance of the spleen had almost completely normalized. One abnormality was present, however; the percentages of B lymphocytes in spleen and lymph nodes were considerably below the unstimulated values.

If multiple injections of GaM6 were given to maintain circulating levels of this antibody the activated immune state persisted. As many as 40% of spleen cells contained intracytoplasmic Ig 13 days after the initial anti-δ injection and histologic sections of spleen demonstrated many lymphoid cells with relatively large amounts of darkly staining cytoplasm in both the red and white pulp.

DISCUSSION

Our data suggest that in vivo B cell activation by soluble anti-δ proceeds through 2 phases. The first, which includes increases in sIg density, cell size, and rate of DNA synthesis appears to be T-independent. The second phase, which includes a further increase in rate of DNA synthesis, acquisition of sIgM, and Ig secretion, appears to be T-dependent and, to a large extent, carrier dependent, which suggests that T cell help specific for goat Ig may be polyclonally focused onto B cells by GaM6 antibody.

Our results have a number of implications. First, they establish the validity of a two stage model of B cell activation under physiological conditions; second, they support the view that B cells acquire sIgG in the process of differentiating into IgG secreting cells. Third, they indicate that in the presence of endogenously generated T cell help, the interaction of a ligand with sIgD can activate B lymphocytes to secrete antibody even when that ligand does not interact with sIgM. As other papers in this volume suggest that the interaction of ligand with sIgD is not required for B cell differentiation into antibody secreting cells, it seems likely that the interaction of ligand with either sIgD or sIgM can lead to B cell terminal differentiation in the presence of appropriate helper factors. This interpretation suggests
that blocking of in vitro immune responses by anti-\(\alpha\) or anti-\(\delta\) antibodies is due to a direct suppressive effect induced by the binding of these ligands to \(\text{Sig}\) or \(\text{Sig}\) rather than to their blocking of antigen binding by \(\text{Sig}\) or \(\text{Sig}\). In previous papers we have presented our belief that an important functional difference between \(\text{Sig}\) and \(\text{Sig}\) is the relative strength of the suppressive effect generated by ligand - \(\text{Sig}\) interaction, with the binding of ligand to \(\text{Sig}\) producing the greater suppressive effect.\(^{19}\)

It is of interest that the anti-\(\delta\) concentrations required to achieve polyclonal activation in vivo and in vitro are higher than the concentration of sub-antigens that are required to induce specific antibody secretion under similar conditions. The low epitope density and helper factor stimulating abilities of soluble anti-Ig antibodies relative to some antigens may contribute to this difference. For example, Parker has found that binding anti-Ig antibodies to an insoluble matrix and adding helper factors to an in vitro culture system substantially reduce the quantities of anti-Ig antibody required for B cell activation.\(^{14}\)

The mechanisms by which T lymphocytes are stimulated to proliferate in the second phase of anti-\(\delta\) activation are not clear. Possibilities include production of factors by activated B cells that induce T cell proliferation, induction of T cell proliferation by goat Ig-mouse anti-goat Ig complexes, and stimulation of an autologous MLR by new antigenic determinants on Ga\(\delta\) modified B lymphocytes.

The reasons for the collapse of the activated immune system once anti-\(\delta\) is catalyzed are also obscure. Histologic data suggest that cells die in situ in the spleen, rather than migrate to other organs. Specific or non-specific suppressor macrophages or T lymphocytes, killer cells specific for activated lymphocytes, and an immunologic network in which polyclonal activation results in secretion of Ig molecules that induce "complementary" suppression by binding to each other's idiotypic determinants may all contribute to the collapse. None of these mechanisms, however, leads inevitably to suppression of the activated immune system, since that state appears to be maintained as long as circulating levels of anti-\(\delta\) are present.

In many ways polyclonal activation by Ga\(\delta\) antibody seems to closely parallel activation of antigen binding clones by a T-dependent antigen. The relative ease with which the in vivo Ga\(\delta\) system provides large populations of activated B and T lymphocytes should therefore be of great value in the study of antigen activated cells and the molecular bases of B lymphocyte activation and regulation.
ACKNOWLEDGEMENTS

We thank Mrs. Shannon Wilburn and Mr. Emeraldo Dace for their expert technical assistance and Ms. Janet Thomson for typing this manuscript. This work was supported by USPHS protocol numbers RO8307, RO8308, CO8410, and the Naval Medical Research and Development Command Work Unit M0995.PH.001.1030. The opinions and assertions contained herein are the private ones of the writers and are not to be construed as official or as reflecting the views of the Navy Department or the naval service at large. The experiments reported herein were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Resources, National Research Council, Department of Health, Education and Welfare Publication (NIH) 78-21.

REFERENCES