CHARACTERIZATION OF SELF-REACTIVE B CELLS
BY POLYCLONAL B-CELL ACTIVATORS*

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Polyclonal B-cell activators (PBA)\(^1\) are substances which directly activate Ig-
positive cells to proliferation and antibody secretion by interacting with recep-
tors that are not the immunoglobulin receptors. Since the activation does not
involve the variable part of the Ig receptors (1) the activation is not immunologi-
cally specific. However this does not necessarily imply that PBA always activate
large populations of B cells. On the contrary it has been demonstrated that
different PBA act on different small subsets of B cells and the type of response
reflects the differentiation stage of the responding B cells (2, 3). Thus dextran
sulfate (DXS) stimulates DNA synthesis to a large extent but little antibody
response, contrarywise purified protein derivate of tubercle bacteria RT32 (PPD)
activates antibody responses but only a low rate of DNA synthesis, whereas
lipopolysaccharide from \textit{Escherichia coli} 055:B5 (LPS) activates both DNA
synthesis and antibody production (2). Other parameters of in vitro induction
such as the age of the donor, the organ origin of the cells, the genetic back-
ground, and the cells density requirements, differs significantly for each PBA
(2, 4).

Since PBA are competent to activate B cells of any immunological specificity
and thus to reveal the total V gene repertoire, they should also trigger resting B
cells with receptors directed against self provided that such cells exist in the
organism. The existence of autoreactive cells was predicted from the "one
nonspecific signal" hypothesis (5) and was confirmed by direct experiments
(references 6 and 7, and footnote 2).

One of the experimental methods used to demonstrate the PBA capacity to
activate self-reactive cells was to inject mice with optimal doses of LPS for
induction of polyclonal response (6). The sera of such animals were collected 3
days after injections and tested for cytotoxic activity against autologus and
syngeneic \(^{51}\)Cr-labeled spleen cells, using rabbit serum as a source of comple-

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\(^1\)Abbreviations used in this paper: BSS, balanced salt solution; DXS, dextran sulfate; LPS,
lipopolysaccharide from \textit{Escherichia coli} 055:B5; NNP, 4-hydroxy-3,5-dinitrophenylacetyl; PBA,
polyclonal B-cell activators; PPD, purified protein derivate of tubercle bacteria RT32; RC', rabbit
complement.

\(^2\)Primi, D., C. I. E. Smith, L. Hammarström, and G. Möller. 1976. Induction of autoantibodies
to autologous and syngeneic spleen cells by LPS. Manuscript submitted for publication.
ment (C). There was consistently a detectable cytotoxic effect, and proper controls showed that cytotoxicity was most likely due to the appearance of self-reacting antibodies produced by nonspecifically activated B cells.

These findings suggested that self-reactive B cells do exist in normal animals and thus that they are not irreversibly tolerized. If this is so it has to be expected that the capacity of these cells to be activated after stimulation with different PBA, would reflect the heterogeneity of all the Ig-positive cells.

In order to investigate this, we injected mice with different PBA (DXS, LPS, and PPD) and studied the C-dependent cytotoxicity caused by sera from injected mice against syngeneic target cells, with regard to the different parameters used for characterization of B-cell subpopulations. The pattern of the response that we found strongly suggested that the capacity of self-reactive cells to release immunoglobulins reflected the polyclonal-activating capacity of the different PBA used and that these responses depend on the same parameters used for differentiation of B-cell subpopulations. The implications of these findings for the understanding of the mechanism of B-lymphocyte triggering and for self-nonself discrimination are discussed.

**Materials and Methods**

**Animals and Immunizations.** A x 5M, A/Sn, A.CA, CBA, and A x C57BL mice, matched for age and sex, were used throughout the experiments. The mice were injected intraperitoneally (i.p.) with varying doses of PBA in a volume not exceeding 0.2 ml. in balanced salt solution (BSS).

**Mitogens.** Three different mitogens were used. These were LPS obtained by phenol water extraction according to the method of Westphal et al. (8) (provided from Karolinska Institutet, Department of Bacteriology, Stockholm, Sweden), PPD (obtained from Statens Seruminstitut, Copenhagen, Denmark) and, DXS (obtained from Pharmacia Fine Chemicals, Ltd., Uppsala, Sweden). When not otherwise stated the concentration of the mitogens used throughout the experiments was 150 μg/animal.

**Sera.** Mice were bled from the retro-orbital plexus usually 4 days after mitogen injections. The sera were separated by centrifugation 1 h later and stored in aliquots at −20°C after being inactivated at 56°C for 30 min.

**Plaque Assay.** Polyclonal antibody production was measured by a modification of Jerne's hemolytic plaque assay using the hapten 4-hydroxy-3,5-dinitrophenylacetyl (NNP) coupled to sheep red blood cells (SRBC). These target cells have been shown to be suitable for detecting polyclonal antibody synthesis (9).

**Reconstitution of Irradiated Animals.** Mice were irradiated with 750 rad and left for 4 h. The animals were then injected with 100 IU heparin and reconstituted intravenously (i.v.) with 2.5 × 10⁶ syngeneic fetal liver cells in a vol of 0.2 ml. of BSS. The mice were protected by 50 μg/ml tetracycline (Dumocyclin) in the drinking water.

**Target Cells.** Spleen cell suspensions from mice were obtained by pressing the spleen through stainless screens. The resultant suspension was pipetted vigorously and the remaining cellular aggregates allowed to settle for 5 min. The cells remaining in the supernate were centrifuged and the pellet was resuspended in a solution made up by one part of BSS and eight parts of 0.82% NH₄Cl in order to lyse the RBC in the suspension. The cells were then washed three times before labeling with °°Cr.

RBC were obtained by bleeding the mice from the retro-orbital plexus and the blood was collected in tubes containing ACD solution in order to avoid clotting. The cells were washed three times before labeling.

**Labeling with °°Cr.** 0.1 ml of sodium chromate (sp act 10 mCi/ml) was added to 10⁶ packed spleen lymphocytes in 0.25 ml of RPMI medium supplemented with penicillin, streptomycin (500 IU/ml) (Flow Laboratories, Irvine, Scotland), and 10% fetal calf serum. When RBC were labeled they were suspended in BSS. The cells were incubated for 75 min at 37°C and agitated every 15
The cells were then washed twice and resuspended to a concentration of \(10^7\) ml in RPMI medium.

**Complement.** Rabbit serum, never stored for more than 1 wk, was used as source of C (usually at a 1/5 dilution in BSS) after absorption according to the method of Boyse et al. (10), using a 30% suspension of thymus and spleen cells.

**Assay for Cytotoxicity.** 50 \(\mu\)l of a cell suspension, containing \(5 \times 10^6\) labeled target cells, was pipetted into hemolysis tubes containing 50 \(\mu\)l of serum from mitogen-injected or control mice. Controls consisted of target cells incubated with serum from untreated syngeneic mice in the presence of C and target cells treated with C or medium alone. To measure total releasable isotope the target cells were incubated with sodium dodecyl sulfate (SDS). The mixtures were incubated for 1 h at 37°C. After two washings in BSS the cells were resuspended in 50 \(\mu\)l of rabbit C (RC) and incubated for another hour at 37°C in a water bath. All assays were done in triplicate. After incubation the tubes were centrifuged and the supernates and pellets counted in a gamma counter. The percentage of \(^{51}\text{Cr}\) released was calculated as follows:

\[
\frac{\text{cpm supernate} - \text{Bg. in counter}}{(\text{cpm pellet} - \text{cpm supernate}) - 2 \text{ Bg. in counter}}
\]

Specific lysis was calculated according to the following formula:

\[
\frac{\% \text{ lysis exp.} - \% \text{ lysis control}}{\% \text{ maximum lysis} - \% \text{ lysis control}} \times 100.
\]

**Results**

**Induction of Cytotoxicity Varies with the PBA Used.** In order to study the C-dependent cytotoxic activity against syngeneic spleen cells of sera from mice injected with different PBA we injected A \(\times\) 5M mice with 150 \(\mu\)g of DXS, LPS, and PPD. The polyclonal antibody response in normal spleen cells after stimulation with these three substances is known to differ substantially. Thus PPD and LPS give a high antibody response, whereas DXS induces a low antibody response (2). Fig. 1 shows the C-dependent cytotoxic response against syngeneic \(^{51}\text{Cr}\)-labeled spleen cells of sera collected 4 days after the injection of each of the three different mitogens. The rabbit sera, used as C source, were absorbed, before testing, with 1/3 of its volume of packed target cells in order to decrease the spontaneous lytic activity. The pattern of the response that we found reflected the different polyclonal-activating capacity of these substances with a maximum isotope release caused by sera of PPD-injected mice and a minimum release caused by sera of DXS-treated animals.

**Strain Dependency of the Lytic Activity.** One of the parameters used to differentiate B-cells subpopulations is the different activating capacity of B-cell mitogens for spleen cells from different strains of mice. We have tested the lytic activity against syngeneic spleen cells of sera from A, A \(\times\) 5M, and CBA mice 4 days after injection of DXS, LPS, and PPD. As can be seen in Fig. 2 all the sera had cytotoxic activity, but the level of activity varied from strain to strain. A \(\times\) 5M as well as CBA are known to respond very well to LPS, whereas A is a low responder. The specific lysis caused by the sera tested reflects this strain dependency not only to LPS, but also to PPD as well as to DXS. This is in accordance with previous experiments in which we could not detect any specific lysis with sera from LPS-injected C3H/HeJ mice, which are genetically nonresponders to LPS, whereas the lytic activity was very high with sera from C3H/Tif mice which are LPS high responders.
DXS Pretreatment Does Not Increase Autoantibody Production after Injection of LPS. It has been reported that administration of DXS to mice, resulted in an enhanced in vitro response to LPS when spleen cells were cultured in vitro 4 days after in vivo prestimulation (3). This led to the conclusion that DXS-activated cells exhibited an increased sensitivity to LPS and that the enhancement of the response caused by DXS pretreatment was due to a direct effect of DXS on the cells that later responded to LPS. In order to study if this enhanced response also occurred with our autoantibody response, we injected A × C57BL/10 mice with 100 µg of DXS. 4 days later we administrated 150 µg of LPS to the pretreated mice as well as to normal animals. Sera were collected 4 days later and tested for C-dependent cytotoxicity against syngeneic spleen lymphocytes. As can be seen in Fig. 3 we did not find any enhancement of the lytic response using sera from DXS-pretreated mice. The failure to reproduce the expected synergism could be due to the migration in vivo of the DXS-activated cells. To study this possibility we tested spleen cells from LPS-injected mice for polyclonal antibody response in the plaque assay. Table I shows that prestimulation with DXS increased to a large extent the polyclonal antibody response to LPS. This result argues against the migration of the DXS-activated cells as the possible cause of lack of synergism in our original experiment. A more likely explanation is that not all the LPS responder cells
A/Sn, A × 5M, and CBA mice were injected with 150 μg of LPS (A), PPD (B), and DXS (C). Sera were collected 4 days later and tested for C-dependent cytotoxicity against syngeneic spleen cells.

Specific lysis of syngeneic spleen cells induced by sera from A × C57BL/10 mice injected at day 0 with 100 μg of BSS (■) or DXS (■). 4 days later both groups were reinjected with 100 μg of LPS or DXS. Sera were collected 4 days after the second injection.

can be preactivated by DXS, it may exist as a subpopulation of LPS responder cells whose precursors are not DXS sensitive.

*Pattern of Response of Polyclonally Activated Precursor Cells.* DXS has been shown to activate precursor B cells, while PPD and LPS lack this capacity (3). In order to study if this pattern could be reproduced when synthesis of self-reactive immunoglobulins is measured, 2.5 × 10⁷ fetal liver cells were injected into syngeneic irradiated recipients and the sera of these animals were tested for their capacity to lyse syngeneic spleens cells after the administration of DXS, LPS, and PPD. Since it has been reported that radioreisitent cells disappear 4 days after irradiation (3), we injected the mitogens at day 6 after reconstitution and the sera were collected 4 days later. We found a significant
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Table I

| Day 0 Prestimulation | Day 4 Activation | Day 8 PFC/10^6 Cells |
|----------------------|------------------|----------------------|
| BSS                  | BSS              | 75 ± 25              |
| DXS                  | DXS              | 240 ± 10             |
| BSS                  | LPS              | 2,296 ± 145          |
| DXS                  | LPS              | 4,000 ± 128          |

The data are presented as the mean ± SE of triplicate experiments. DXS injected 100 μg/animal. LPS injected 100 μg/animal.

Figure 4. Specific lysis caused by sera from A × C57BL/10 mice collected at different times after irradiation and reconstitution with fetal liver cells and injection of 100 μg of LPS, PPD, and DXS. Fig. 4A represents the specific lysis caused by sera from normal animals injected with the same PBA; Fig. 4B represents the specific lysis caused by sera of mice irradiated and reconstituted and injected 6 days after reconstitution; in Fig. 4C the mice were injected 15 days after reconstitution.

decrease in the specific lysis using these sera as compared with sera from normal mice injected with the same PBA. However a certain degree of cytotoxic activity was still detectable (Fig. 4). To find the cause of this residual response we tested spleens of reconstituted mice for polyclonal response. As can be seen in Table II, 6 days after irradiation there was still a significant polyclonal response, thus excluding the possibility of complete elimination of radioresistant cells. When the PBA were injected at day 15 after reconstitution and the sera collected 4 days later, we found that the cytotoxic activity against syngeneic spleen cells of these sera returned to normal levels. This is in perfect agreement with the sequential acquisition in the PBA responsiveness in the differentiation process as described by other authors (2, 11, 12).

Involvement of the Histocompatibility Antigens in the Lysis of the Target Cells. We have shown that the sera of mitogens injected mice contain antibodies directed against syngeneic cells. These autoantibodies are probably released from polyclonally activated B cells that are programmed to react against self. It is of interest to determine the specificity of these antibodies with particular attention to the possibility that they are directed against the
TABLE II
Spontaneous PFC against NNP-SRBC from Spleen Cells 2 and 5 Days after Animals Irradiation

| Animals condition       | PFC/10^6 spleen cells |
|-------------------------|-----------------------|
|                         | IgM       | IgG       |
| Normal animals          | 23.7      | 3.9       |
| Day 2 after irradiation | 35.0      | 7.2       |
| Day 5 after irradiation | 156.0     | 126.7     |

Fig. 5. C-dependent cytotoxicity caused by sera from A/Sn mice collected 4 days after injection of 150 μg of PPD. The same sera were also absorbed, before testing, on 1/3 volume of target cells and on the same volume of spleen cells from A.CA mice.

major histocompatibility antigens, or if they are capable of reacting with a variety of other surface antigens of the target cells as well.

To study this we injected A/Sn (H-2^a) mice with 150 μg of PPD and collected the sera 4 days later. The sera were tested in the cytotoxic assay using the same sera absorbed with syngeneic spleen cells as control. Before testing, some of the sera were also absorbed with spleen cells from the co-isogenic strain A.CA (H-2^b). Fig. 5 shows that absorption of the sera with these two kinds of cells eliminated the cytotoxic activity of the sera to the same extent. Since the only difference between the strain A/Sn and A.CA is in the H-2 region we conclude that the self-reactive immunoglobulins produced by polyclonally activated B cells are directed against a variety of surface antigens on the target cells and that the anti-H-2 antibodies represent, if they exist, such a small part of the antibodies that no detectable cytotoxicity remains. Sera from PPD-injected mice were capable of lysing syngeneic RBC in the presence of C (Fig. 6). Since RBC have a low concentration of H-2 antigens compared to
Discussion

Our results indicate that the different activating properties of PBA are detectable also when synthesis of self-reactive immunoglobulins is measured. DXS stimulates the production of autoantibodies to a relatively low extent, whereas LPS and PPD induce a higher degree of activation of self-reactive B cells. We have found a higher level of specific cytotoxicity in the sera of those mice that, for genetic reasons, respond better to LPS compared with those that respond less well. We detected a diminished level of autoantibodies in the sera of mice that have been irradiated and reconstituted with fetal liver cells before injection of LPS and PPD. However, when these PBA were injected after a period that allowed the injected cells to mature, the sera of these animals recuperated the lytic activity against syngeneic cells. This suggests that PPD and LPS are incompetent to activate immature B cells. It was not possible for us to enhance the response of LPS by pretreating the mice with DXS. However, when measuring the polyclonal response it was found that DXS pretreatment increased the number of plaques to NNP-SRBC after LPS injection. This implies that all DXS-responding cells are not necessarily the precursors of the later LPS-responding cells.

These findings suggest that self-reactive B cells exist in a resting state in normal animals and that they can become activated by PBA to secrete their gene products in the same way as all B cells. From this it follows that self-nonself discrimination must be carried out at the T-cell level and that tolerance does not exist at the B-cell level.

Our findings have implications for the understanding of the mechanism of
lymphocyte triggering. The two-signal hypothesis (13) states that the interaction between the antigen and the Ig receptors results in an irreversible state of immunological tolerance. This concept predicts that when a cell programmed to react against a self-antigen becomes immunologically competent it has to become irreversibly tolerized, because the self-antigen will provide the tolerogenic signal "one". The Ig-positive cells responding to PPD and LPS have been shown to be mature B cells and therefore it has to be expected that no self-reactive B cells would exist in this population, since these cells would all have met self-antigens during their differentiation pathway. Obviously this expectation was not fulfilled by the findings. Another consequence of the concept is that the tolerizing event should depend only on the interaction between the antigen and the Ig receptor. This should occur in all the Ig-positive cells irrespective of the genetic background. However, we have found that autoantibody production is strain dependent in a way that cannot be explained by the two-signal concept.

Instead our findings are in accordance with the one nonspecific signal hypothesis (5). This assumes that only one nonspecific signal activates B cells and this signal is delivered by nonclonally distributed receptors for PBA. Therefore PBA activate cells of all specificities, including those directed against self. The prevention of the formation of autoantibodies in normal conditions is probably due to the absence of self-reactive T cells. Since all autologus antigens are thymus dependent, self-recognizing B cells cannot normally be activated, due to the lack of cooperation between B and T cells. This theory is strongly supported by the recent demonstration that tolerance to a hapten protein conjugate did not effect the B cells, which could be activated to antibody synthesis against the tolerogen by LPS (14).

In conclusion our findings demonstrate that self-tolerance does not exist at the B-cell level and that self-reactive cells, as well as cells of all other specificities can be activated by PBA in different ways according to their activating capacity, thus excluding that contact between Ig receptors and self-antigens causes irreversible tolerance.

Summary

The existence of autoreactive B cells was predicted by theoretical considerations and, recently, confirmed by direct experiments. The aim of the present work was to investigate if the capacity of self-reactive B cells to be activated with different polyclonal B-cell activators (PBA) reflects the heterogeneity of the response as seen in all the Ig-positive cells.

We injected mice with dextran sulfate, lipopolysaccharide from *Escherichia coli* 055:B5, and purified protein derivate of tubercle bacteria RT32 and studied the complement-dependent cytotoxicity against syngeneic spleen cells caused by the sera from injected mice with regard to the different parameters used for characterization of B-cell subpopulations. It was found that the capacity of self-reactive B cells to secrete antibodies reflects the polyclonal-activating capacity of the PBA used. The implications of these findings for the understanding of the triggering mechanism of B lymphocytes and for self-nonself discrimination are discussed.
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