EXPERIMENTAL CONDITIONS FOR OBTAINING SUPPRESSOR AND HELPER EFFECTS ON THE PRIMARY IN VITRO IMMUNE RESPONSE BY LYMPHOCYTES ACTIVATED BY POLYCLONAL T-CELL ACTIVATORS

BY LUIGI NESPOLI, GÖRAN MÖLLER, DOUGLAS WATERFIELD, AND RICHARD EKSTEDT

(From the Division of Immunology, Karolinska Institute, Wallenberg Laboratory Lilla Freskåts, 104 05 Stockholm 50, Sweden)

It is now well established that T lymphocytes function as helper cells in the induction of immune responses to thymus-dependent antigens, but they can also exert suppressive effects on the same responses. Attempts have been made to ascribe these two effects to distinct T-cell subpopulations using irradiation sensitivity, differing sedimentation rates, and cell surface markers as tools (2, 5, 8, 14, 16). Most of these studies have been interpreted to indicate the existence of at least two distinct subsets of T cells (helper and suppressor cells).

The selective T mitogens phytohemagglutinin (PHA)\(^1\) and concanavalin A (Con A) are capable of inducing both helper and suppressor activities, and are therefore suitable tools for the analysis of the mechanism of action of suppressor T cells (4, 13, 15). It has been shown that there are at least two T-cell targets for the action of Con A. One is shortlived, radiosensitive, susceptible to anti-T-cell antisera, and mediates the inhibitor effect (4, 5). The other subpopulation is responsible for the stimulatory activity and is radioresistant, long-lived, relatively insensitive to complement-mediated lysis in the presence of anti-T sera (4, 5). Using the Ly phenotypes and Con A-activated cells, it has been shown that the Ly 23 but not the Ly 1 lymphocytes can suppress the antibody response (2, 7, 8). In primary and secondary responses to sheep red blood cells (SRC), the cells of the Ly 2\(^+\), in particular Ly 23 cells, have a suppressive activity (2, 7, 8). Helper and suppressor T lymphocytes have also been separated on a Ficoll velocity sedimentation gradient after stimulation with Con A in vitro. The population of blast cells exhibits suppressive activity, while the small cells mediate helper effects (16). The other T-cell mitogen used (PHA) can also either suppress or enhance the in vitro primary immune response of mouse spleen cells to heterologous erythrocytes (13). Con A added in vitro can induce helper or suppressor cells in a primary anti-SRC response, depending on the dose and the

---

\(^1\) Abbreviations used in this paper: BSS, balanced salt solution; Con A, concanavalin A; FCS, fetal calf serum; LPS, lipopolysaccharide; NNP, 4-hydroxy-3,5-dinitrophenylacetyl; PBA, polyclonal B-cell activator; PFC, plaque-forming cells; PHA, phytohemagglutinin; PPD, purified protein derivative; PTA, polyclonal T-cell activator; SRC, sheep red blood cells.
The aim of the present experiments was to analyze the helper and suppressor effects on the primary immune response to SRC of T cells activated by the polyclonal T-cell activators (PTA) Con A and PHA with particular emphasis on the nature of the target cell and the experimental conditions leading to the appearance of helper versus suppressive effects.

We will show that helper and suppressor activities induced by Con A and PHA in the in vitro primary anti-SRC response are dose-dependent phenomena, but the same concentration of a PTA can induce help or suppression depending on the experimental conditions. Finally it will be shown that suppressor cells do not act directly on B lymphocytes.

Materials and Methods

**Mice**  Both male and female mice from 4–12 wk of age of the C3H/Tlf and B10.5M were used in all the experiments reported with comparable results. The animals were housed not more than 10 per cage and allowed food and water ad libitum.

**Antigen and Mitogens**  SRC, obtained from the same donor, were stored at 4°C in Alsever's solution. They were washed three times in balanced salt solution (BSS) before use. Con A was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, as a lyophilized powder purified by Sephadex chromatography and containing less than 0.1% carbohydrate. PHA was obtained from Wellcome Reagents Limited, England, as a sterile, freeze-dried protein fraction of selected Phaseolus seed extract in which the mitogenic/hemagglutinating activity ratio has been increased by a factor of about 100:1 during purification. It was dissolved at 1 μg/ml concentration in BSS and diluted before using in the same medium.

Lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 was extracted by Prof. T. Holme (Dept. of Bacteriology, Karolinska Institute, Stockholm) by the phenol-water method and obtained as a lyophilized powder. Purified protein derivative (PPD) from Statens Seruminstitut, Copenhagen, Denmark, was free of preservatives and contained 1 mg/ml of PPD.

**Cell Culture Systems**  Spleens from the mice were removed aseptically and pressed through sterile 60-mesh stainless steel screens into BSS. The cells were resuspended by repeated pipetting and large clumps allowed to settle. The resulting cell suspensions were washed three times in sterile BSS, resuspended after the final wash in medium as described by Mishell and Dutton (9) supplemented with 5% FCS at a concentration of 1 × 10^7/ml. Cell counts were made with a Burker hematocytometer, and viability was tested by the trypan blue exclusion method.

Cultures for induction of primary immune responses were performed in Mishell-Dutton medium supplemented with 5% fetal calf serum (FCS) (Rehatuin, Armour Pharmaceutical Co., Chicago, Ill.) 10 million spleen cells in 1 ml were incubated at 37°C in 3-cm Petri dishes (Nunc, Roskilde, Denmark) in plastic boxes as described before (3) for 4 days. SRC were added at the beginning of the culture, at a final concentration of 0.025%.

The mitogens, Con A and PHA, at the desired doses were added at different times as indicated in the different experiments. The cultures were done in triplicate, and the plaque-forming cell response (PFC) was assayed as described previously (1), with the exception that the mixtures were plated in triplicate as a single spot spread in the bottom and top of 9-cm diameter plastic Petri dishes.

**Fetal Calf Serum**  Two different batches were used in our experiments. Batch no. N 50003 which was able to substan a high response to SRC is referred to in the text as "good FCS", whereas the batch no L 25612, which only supported a low level of response, is referred to in the text as "bad FCS."

Ekstedt, R. D., J. D. Waterfield, L. Nespoli, and G. Moller. 1976. Mechanism of action of suppressor T cells. In vivo Con A activated suppressor T cells do not directly affect B cells. *Scand J Immunol.* In press
FIG. 1. Effect of Con A and PHA added at the same time as the antigen on the primary immune response to SRC in good fetal calf serum. (■—■) indicates the response after addition of Con A and (×—×) the response after addition of PHA. Background (▲) and the primary anti-SRC response (△) determined at day 4 are also indicated.

Results

Effects of PTA on Primary Anti-SRC Responses in Good FCS

A batch of FCS which makes it possible to induce a strong response to SRC in the Mishell-Dutton in vitro system is defined as a "good" serum.

Effect of PTA added at initiation of the cultures. When the culture media was supplemented with good FCS, there was an expected high primary response to SRC when the antigen alone was added to the cultures. The simultaneous addition of SRC and PTA resulted in a two to threefold increase of the number of PFC (Fig. 1). The strongest enhancement of the specific anti-SRC response was observed with submitogenic concentrations of the PTA (0.25 μg/ml), which by themselves induce a very low T-cell response as measured by DNA synthesis. This is in agreement with previous reports showing an additive effect between antigens and mitogens. The response decreased with increasing PTA concentration and very marked suppressive effects occurred with 1-2 μg/ml. These concentrations are suboptimal for Con A, which activate T cells optimally at 5 μg/ml in the presence of serum.

Thus, in these experimental conditions, low concentrations of PTA, which activate few T cells, resulted in marked helper effects and a considerably stronger PFC response to SRC. With increasing PTA concentrations the suppressive effects became apparent.

Mitogens added after 24 h of culture. When PTA were added after 24 h of culture the dose-response profile changed markedly as compared to that obtained when PTA was added at initiation of the cultures. Thus, an enhanced anti-SRC response still occurred, but now at much higher concentrations (2-4 μg/ml) of Con A, which is close to the optimal concentration for T-cell activation, as measured by DNA synthesis. In contrast to the previous findings there was
no sign of suppression by any concentration of Con A. The small suppression observed with 4 \( \mu g/ml \) of PHA is probably caused by toxic effects, since this concentration is superoptimal, 1–2 \( \mu g/ml \) being optimal (Fig. 2).

Thus, after 24 h, suppressor effects could not be induced by the addition of PTA to the cultures, whereas helper effects were still observed, although higher concentrations of the PTA were required than when it was added at time zero.

**Effects of PTA on Primary Response to SRC in “Bad” FCS**

A batch of FCS that cannot support induction of a primary immune response to SRC at all or very weakly is referred to as "bad.”

**Effect of PTA added before or at the same time as the antigen.** When the mitogens were added to the cultures before addition of SRC, the results shown in Fig. 3 were obtained. As can be seen, there were virtually no suppressive effects, whereas a marked enhancement of the response occurred with most PTA concentrations, peaking at suboptimal concentrations for T-cell activation. Analogous results were obtained with PTA added at the same time as SRC (Fig. 4).

**Effect of PTA added after the antigen.** A delay of 12–24 h between addition of SRC and PTA had two main effects (Figs. 5 and 6): the number of PFC to SRC increased with time of delay, and the PTA concentrations needed to obtain the enhanced response approached those optimally activating for T cells. There were no suppressive effects with any PTA concentration when the PTA were added 24 h after SRC.

It follows from these results that the addition of PTA to cultures in bad FCS restores the ability of the system to support a primary immune response to SRC. Presumably good sera already contain those factors that are provided by activated T cells.

**Primary Immune responses to SRC in Serum-Free Medium in the Presence of Con A.** It has been shown before (3) that primary immune responses to SRC cannot be induced in serum-free medium unless nonspecific T- or B-cell activa-
Fig. 3. Effect of Con A and PHA added 6 h before the antigen on the primary immune response to SRC in bad fetal calf serum Symbols as in Fig. 1.

Fig. 4. Same as in Fig. 3, but the PTA added at the same time as the antigen
extensively. These preactivated cells were thereafter added to syngeneic untreated spleen cells cultivated in 10% FCS not competent to support a primary immune response to SRC. As can be seen in Fig. 8 the addition of increasing numbers of PTA-activated cells to these cultures resulted in progressively stronger anti-SRC immune responses, which reached expected levels for a primary response in good batches of FCS. The PTA pretreated cells themselves showed increased numbers of PFC against SRC, as expected from polyclonal T-cell activation.

PTA Do Not Suppress LPS-Induced Activation. To study the influence of PTA on a thymus-independent polyclonal response, we added different concentrations of PTA to B cells preactivated by LPS.

Mouse spleen cells (10⁷ cells/ml) were cultured for 6, 12, and 24 h with 100 μg
Fig. 7. Effect of Con A on the primary immune response to SRC in serum-free medium. Solid lines indicate the response in the presence and dotted lines in the absence of SRC. Con A was added at the same time as the antigen and the response determined at day 4.

Fig. 8. Effect of PTA-pretreated lymphocytes on the primary immune response to SRC in bad fetal calf serum. Solid lines indicate the response in the presence and dotted lines in the absence of SRC. Both Con A- (■) and PHA- (×) pretreated lymphocytes were used. PTA-pretreated lymphocytes and the antigen were added simultaneously and the response determined at day 4.

of LPS/ml. Thereafter, the lymphocytes were harvested, extensively washed, and the number of viable cells determined by the trypan blue exclusion test. Subsequently the cells were recultured at the same cellular density in the presence of different concentrations of PTA. As controls, spleen cells were cultured in the absence of PTA. The number of anti-NNP PFC was evaluated 48 h after addition of PTA.

In contrast with what was observed in the thymus-dependent response to SRC, the polyclonal B-cell activation was not affected by the addition of PTA doses ranging from 0.125 to 0.5 µg/ml, while doses of 1 µg partially decreased the PFC number when added to cells pretreated for 6–12 h with LPS (Figs. 9 and 10). If LPS activation was prolonged for 24 h, there was no suppression of the
polyclonal response induced by LPS (Fig. 11). We can therefore conclude that PTA did not significantly suppress the direct B-cell activation induced by LPS. The decreased PFC responses per culture with superoptimal PTA concentrations are most likely due to toxicity (11).

**PTA-Activated Cells Do Not Suppress PBA-Induced Activation.** To study whether PTA-activated lymphocytes could suppress polyclonal B-cell activation by LPS and PPD two experimental systems were employed. In the first, increasing numbers of PTA-preactivated cells were added to untreated syngeneic spleen
cells, and the cultures were stimulated with optimal concentrations of LPS or PPD. The polyclonal antibody response was measured at day 2 against NNP-coated SRC. The second system was similar, except that the number of cells per culture was kept constant. Thus, different proportions of PTA-activated cells were mixed with untreated cells, and the mixture was stimulated with LPS or PPD. In both systems pretreatment was performed by activating spleen cells in serum-free medium with 0.5 μg/ml of Con A or 1 μg/ml of PHA for 24 h. The cells were thereafter harvested and carefully washed. As can be seen in Figs. 12–14 both systems gave the same results. There was no indication that PTA-
pretreated lymphocytes could exert any suppressive effect on LPS- or PPD-induced polyclonal activation.

Discussion

The addition of Con A or PHA to lymphocyte cultures can cause suppression of primary immune responses to SRC. Also, cells preactivated by these PTA have suppressive effects. It is clear from these studies as well as others (7, 8, and footnote 2) that suppressor T cells do not act directly on B cells, since neither thymus-independent specific immune responses² or polyclonal activation caused by PBA could be suppressed by optimal or below optimal concentrations of PTA or by any number of preactivated cells added to the culture. Suppressor T cells must therefore exert their effect on other cell types necessary for induction of primary immune responses to thymus-dependent antigens, such as macrophages or helper T cells. Recent studies suggest that at least in one system (allotype suppression) the target for suppressor T cells is helper T cells (7, 8). Also, Con A-activated cells can suppress the generation of cytotoxicity T cells (12).

An important conclusion from these studies is that the experimental conditions are of major importance in determining whether suppressor effects will be obtained or not. As shown before (4, 5, 15), the time of addition of PTA markedly
influence suppressor and helper effects. Con A or Con A-activated cells make a primary immune response possible in serum-free medium or in bad batches of FCS. Consequently, PTA stimulated the formation of helper cells during these experimental conditions. However, exactly the same procedures resulted in the appearance of suppressor effects when good batches of FCS were used. Furthermore, it was consistently observed in all experimental systems that low concentrations of the PTA employed caused an enhanced response, whereas suppressor effects required higher concentrations (5, 15). It should be noted that a particular concentration of Con A in one experimental system induced helper effects and in another suppressive effects. Taken together, these findings do not easily fit the notion of a particular subset of suppressor T cells being distinct from helper T cells. If a subset of suppressor T cells exists it is certainly very elusive. The outcome of a particular experiment was found to be highly predictable, but the prediction could not be based on the existence of subsets of suppressor and helper T cells. However, one variable stands out as having high predictive value; namely the strength of the specific immune response in control cultures, not given PTA or PTA-activated cells. If there was a high specific immune response to SRC the addition of PTA caused suppression at low concentrations. If the control response was very low, the addition of the same substances or cells at the same concentration induced helper effects.

It seems likely, therefore, that suppression or enhancement of a primary immune response reflects phenomena, not necessarily mediated by distinct cell subpopulations. The explanation most compatible with these findings is that suppressor and helper cells belong to the same T-cell subpopulation. Helper
effects are seen when the culture conditions require more help, and suppressor
effects are observed when adequate help is already provided by the system in
agreement with the findings of others (6, 15, and footnote 2). Therefore, these
findings are compatible with the notion that suppression is due to too much
help.

This conclusion does not contradict the possibility of the existence of suppressor
cells as a distinct and identifiable T-cell subpopulation in other test systems
(for review see 11). However, the primary immune response to SRC has been a
commonly used method for the study of suppressor cells and our findings should
cautions against far reaching conclusions obtained in this system, unless precau-
tions have been taken to test the effects in a variety of experimental conditions.

Summary
The effect of the polyclonal T-cell activators (PTA) Con A and PHA on the
specific immune response to sheep red blood cells (SRC) was studied. Addition of
PTA either enhanced or suppressed the anti-SRC response, and two variables
were found to affect the results: time of addition of the PTA and the strength of
the response in control cultures not given PTA. If the response was high, even
suboptimal PTA concentrations induced suppressive effects, but if the control
response was low, due to deficient batches of sera or because of the absence of
serum, the addition of PTA increased the response or restored it to normal
levels.

Suppression could be obtained if the PTA were added before or at the same
time as the antigen and required high (optimal) PTA concentrations. If addition
was delayed for 12-24 h the suppressive effects disappeared and previously
suppressive concentrations of the PTA now caused an enhanced response.

Analogous results were obtained if preactivated lymphocytes were added to
the cultures instead of soluble PTA. Neither Con A, PHA, or lymphocytes
preactivated by these PTA suppressed the polyclonal response induced by LPS
or PPD.

Irrespective of the time of addition and the culture conditions, enhancement of
the anti-SRC response occurred at lower PTA concentrations than suppression.

It was concluded that suppressor T cells, if they exist, do not act on B cells, but
rather on helper cells needed for induction of thymus-dependent responses. The
findings in this system are not compatible with the existence of a specific subset
of suppressor T cells, but rather with the notion that suppression is caused by
too much help.

We want to thank Miss Susanne Bergstedt for her skillful technical assistance

Received for publication 25 October 1976.

References
1. Bullock, W., and E. Moller. 1972. Spontaneous B cell activation due to loss of normal
mouse serum suppressors. Eur. J. Immunol. 2:514.
2. Cantor, H., F. W. Shen, and E. A. Boyse. 1976. Separation of helper T cells from
suppressor T cells expressing different Ly components. II. Activation by antigen:
after immunization antigen-specific suppressor and helper activities are mediated by
distinct T-cell subclasses. J. Exp. Med. 143:1391.
3. Coutinho, A., and G. Möller. 1973. In vitro induction of specific immune responses in the absence of serum: requirement for non-specific T or B cell mitogens. *Eur. J. Immunol.* 3:351.

4. Dutton, R. W. 1972. Inhibitory and stimulatory effects of concanavalin A on the response of mouse spleen cell suspensions to antigen. I. Characterization of the inhibitory cell activity. *J. Exp. Med.* 136:1445.

5. Dutton, R. W. 1975. Suppressor T cells. *Transplant. Rev.* 26:39.

6. Eardley, D. D., M. O., Staskawicz, and R. K. Gershon. 1976. Suppressor cells: dependence on assay conditions for functional activity. *J. Exp. Med.* 143:1211.

7. Herzenberg, L. A., K. Okumura, H. Cantor, W. L. Sato, S. W. Shen, E. A. Boyse, and L. A. Herzenberg. 1976. T-cell regulation of antibody responses: demonstration of allotype-specific helper T cells and their specific removal by suppressor T cells. *J. Exp. Med.* 144:330.

8. Jandin, J., H. Cantor, P. V. Tadakuma, D. L. Peavy, and C. W. Pierce. 1976. Separation of helper T cells from suppressor T cells expressing different Ly components. I. Polyclonal activation: suppressor helper activities are inherent properties of distinct T-cell subclasses. *J. Exp. Med.* 143:1382.

9. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med.* 126:423.

10. Möller, G. 1975. Suppressor T lymphocytes. *Transplant. Rev.* 26.

11. Möller, G. 1976. Off signals in lymphocyte activation. *Scand. J. Immunol.* 5:583.

12. Peavy, D. L., and C. W. Pierce. 1974. Cell-mediated immune responses in vitro. Suppression of the generation of cytotoxic lymphocytes by concanavalin A and concanavalin A-activated spleen cells. *J. Exp. Med.* 140:356.

13. Rich, R. R., and C. V. Pierce. 1973. Biological expression of lymphocyte activation. I. The effects of phytohormones on antibody synthesis in vitro. *J. Exp. Med.* 137:205.

14. Scavulli, J., and R. W. Dutton, 1975. Competition between Con A-induced stimulatory and inhibitory effects in the in vitro immune response to antigen. *J. Exp. Med.* 141:524.

15. Sjoberg, O., G. Möller, and J. Andersson. 1973. Reconstitution of immunocompetence in B cells by addition of concanavalin A or concanavalin A-treated thymus cells. *Clin. Exp. Immunol.* 13:213.

16. Tse, H., and R. W. Dutton. 1976. Separation of helper and suppressor lymphocytes on a Ficoll velocity sedimentation gradient. *J. Exp. Med.* 143:1199.