Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 2375–2378, April 1982
Immunology

Interactions between molecules (subfactors) released by different T cell sets that yield a complete factor with biological (suppressive) activity

(T suppressor factors/contact sensitivity)

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Contributed by Richard K. Gershon, December 31, 1981

ABSTRACT **T cells that have been immunized to express optimal levels of contact hypersensitivity upon adoptive transfer to normal animals can be inhibited from doing so by incubating them with an antigen-specific T suppressor factor. This factor is composed of at least two subunits which come from cells expressing different Ly phenotypes; an antigen-specific antigen-binding "subfactor" is made by an Ly-1 cell and a non-antigen-binding one is made by an Ly-2 cell. Neither of these cells nor their products express detectable amounts of major histocompatibility gene products. The mode of immunization plays an important role in determining which of these subfactors will be produced. Painting the skin with a reactive hapten immunizes Ly-1 cells that secrete antigen-binding material, whereas intravenous injection of trinitrobenzenesulfonic acid activates Ly-2 cells to produce a second subunit that does not see antigen. There is reason to believe that the molecule that does not bind to antigen does have some antigen specificity. An analysis of the data at hand suggests that the antigen specificity stems from an interaction of the two subunits described with yet another subunit and that biological activity is dependent upon three macromolecules. Thus, the complex level of cellular interactions that regulate immunity may also be reflected in a similar type of complexity in the interactions between their biologically active cell-free products.**

Antigen-specific suppressor T cells play an important role in immunoregulation. It has been found in studies of *in vitro* antibody responses that the activity of these cells is not autonomous. They require help from a special T helper subset that can be identified by its expression of a unique profile of cell surface alloantigens (Ly-1$^+$; Ly-2$^-$; I-J$^+$; Qa-1$^+$) to express optimal effector activity (1). The suppressor effector cells also express a cell surface phenotype (Ly-1$^-$; Ly-2$^+$; I-J$^\pm$; Qa-1$^+$) by which they can be identified (2). Thus, some cells act as inducers of antigen-specific suppression while others act as effector cells. The biological activities of both types of cells can be replaced by substances that they release (3, 4). These collections of biologically active molecules have been referred to as factors. The factor made by Ly-1 inducer cells consists of two macromolecules: one that sees antigen and another that expresses the I-J marker (5). Both molecules come from Ly-1 cells and can be shown to associate with one another in their biologically active form. Biologically active products of somatic cell hybrids have also been shown to be composed of two similar chains (6, 7). However, not all suppressor molecules have been demonstrated to consist of two subunits that can be separated and identified (8).

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A series of suppressor factors that can inhibit contact sensitivity (CS) reactions to reactive haptens has been described (8, 9–11). Some of these appear to act on the "afferent" arm of the immune response in that they prevent immunization, and another group appears to act at the "efferent" arm. The latter group of cells can inhibit the functions of immune effector cells. The cell surface phenotypes of the cells responsible for suppressing contact hypersensitivity responses have not been as well characterized as the suppressor factors that inhibit antibody responses as described above.

One hapten-specific product that inhibits the "efferent" arm of the CS response to picryl chloride (PCl, 2-chloro-1,3,5-trinitrobenzene), described by Asherson and Zembala in 1974 (12), has been studied extensively in our laboratory (13, 14). We have found that the optimal way to produce this factor is to inject mice intravenously with 2,4,6-trinitrobenzenesulfonic acid (TNBSA) and subsequently to paint their flank skin with PCl. The cultured spleen and lymph node cells of mice so immunized release significant amounts of efferent suppressor factor.

We have attempted to determine why two separate forms of antigen inoculation are required to get optimal suppressor factor produced and to characterize the cells responsible for the factor's biological activity. Unlike any suppressor factor heretofore described, the biological activity of this particular factor is dependent upon an interaction of two macromolecules made by cells of different Ly phenotypes. Painting the flank skin with PCl activates an Ly-1$^+$; Ly-2$^-$; I-J$^-$ cell, which releases an antigen-specific product that can be isolated on hapten affinity columns. This molecule has no biological activity that we can measure; however, if it is mixed with the cell-free products of cultured spleen and lymph node cells from mice immunized with TNBSA it acquires potent suppressive activity. The cell responsible for the production of the second macromolecule appears to be antigen specific, although its product does not bind to antigen with a measurable avidity. The cell itself expresses the phenotype of suppressor effector cells (e.g., Ly-1$^-$; Ly-2$^+$; I-J$^-$).

MATERIALS AND METHODS

Animals. CBA/J mice (6- to 8-week-old males) were obtained from the Jackson Laboratories and maintained in the Department of Comparative Medicine, Yale University.

Abbreviations: CS, contact sensitivity, PCl, picryl chloride; TNBSA, 2,4,6-trinitrobenzenesulfonic acid; Ox, 4-ethoxymethylene-2-phenyloxazolone; TsF, T suppressor factor; BGG, bovine gamma globulin; TNP, 2,4,6-trinitrophenyl; MHC, major histocompatibility complex.

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Materials. Bovine gamma globulin (BGG) was purchased from Sigma; TNBSA was obtained from Eastman, PCl was from Chemtronix (Swannanoa, NC) and was recrystallized twice from methanol prior to use. 4-Ethoxymethylene-2-phenyloxazolone (Ox) was from British Drug House (Gallard–Schlessinger, Carle Place, NY) and was recrystallized from methanol/water prior to use.

Anti-Lyt-1.1 and anti-Lyt-2.1 monoclonal antibodies were kindly supplied by F. W. Shen. The anti-Lyt-1.1 was diluted 1:200 and 1.0 ml was used to treat 10^7 cells. The anti-Lyt-2.1 was diluted 1:80 and used the same way. Normal rabbit serum, screened for background cytotoxicity, was used as the source of complement for cytotoxicity treatments with anti-Ly reagents.

Production of Factors. TNBSA-elicited T suppressor factor (TNBSA TsF) was produced by intravenous injection of 0.35 ml of 10% solution of TNBSA in distilled water (pH 7.2 with sodium hydroxide) at days 0 and 4; 3 days later single-cell suspensions were prepared from spleen and peripheral lymph nodes and cultured *in vitro* at a cell density of 1.5×10^7 cells per ml for 48 hr at 37°C in serum-free RPMI medium supplemented with glutamine and an antibiotic mixture. Cells were sedimented at 2500 rpm for 10 min in a Sorvall centrifuge and supernatants were stored at −70°C until used.

PCl TsF was prepared by painting all four paws and the skin of the clipped abdomen with 0.15 ml of a 5% solution of PCl in an ethanol/acetone mixture, 3:1 (vol/vol). The cells of these mice were taken 1 to 4 days after sensitization and cultured as above. In some experiments, before being cultured *in vitro*, cells were treated with antisera against cell surface antigens.

An Ox TsF (equivalent to PCl TsF) was prepared by painting the skin of mice with 0.15 ml of a 3% solution of Ox in an ethanol/acetone mixture, 3:1 (vol/vol), in the same fashion as PCl was applied.

An Ox equivalent to TNBSA TsF was prepared by injecting mice intravenously with 1×10^8 mouse erythrocytes to which Ox was conjugated at days 0 and 4, and these mice were then treated in the same fashion as were the mice that made the TNBSA TsF.

Adoptive Transfers. Mice were skin sensitized with PCl or Ox and after 4 days spleen and lymph node cells of these animals were injected intravenously into naive recipients ($5–6 \times 10^7$ cells per mouse), which were immediately challenged on the ears with corresponding antigen (0.8% PCl or Ox in olive oil). CS responses are expressed as the 24-hr increment in ear thickness [measured by an engineer's micrometer (Mitotope)] in units of 10^{-3} cm. Before being injected, the transfer cells were incubated in different supernatants of their mixtures for 45 min at 37°C. Generally 5×10^7 cells were incubated in 2.5 ml of a particular supernatant.

Affinity Columns. For some experiments cell culture fluids were treated with hapten coupled to BGG-Sepharose 4B. BGG was coupled to Sepharose 4B (Pharmicia) by using the CNBr procedure of Axen *et al.* (15). 2,4,6-Trinitrophenyl (TNP) and Ox were coupled to BGG-Sepharose as reported (14). Bound molecules were eluted from hapten affinity columns by using hapten coupled to ε-aminocaproic acid (0.05 M) in borate/saline buffer (0.1 M, pH 8.4) as reported (14).

RESULTS

Two Types of T Cell Subsets Must Interact to Produce T Suppressor Activity. The results in Table 1 come from an experiment in which individual mice were immunized either with PCl by skin painting or with TNBSA by intravenous injection. Their spleen and lymph node cells were harvested and treated with either normal mouse serum or anti-Ly reagents and complement. The treated cells were mixed together and added to immune cells; the cell mixtures were then transferred to adoptive recipients, which were tested for the expression of CS immediately after cell transfer.

Table 1. Cellular requirement for suppressing the adoptive transfer of CS to normal mice

Group	TNP-immune cells mixed with cells from mice immunized with*		CS response in adoptive recipients, % of control†
	PCl	TNBSA	
1	NMS	–	95
2	–	NMS	119
3	NMS	NMS	19 (22, 0, 40, 16)
4	Anti-Ly-2	NMS	14 (20, 0, 23, 0, 25)
5	Anti-Ly-1	NMS	84 (83, 85)
6	NMS	Anti-Ly-2	73 (80, 67)
7	NMS	Anti-Ly-1	30 (41, 13, 37)
8	Anti-Ly-2	Anti-Ly-1	49 (70, 61, 17)
9	Anti-Ly-1	Anti-Ly-2	100 (102, 98)

* Some mice were immunized in a way that leads to the production of PCl TsF and others in a way that yields TNBSA TsF. These *cells* were mixed with TNP-immune cells, incubated for 1 hr, and then injected into normal recipients. The cells were treated with either normal mouse serum (NMS) or anti-Ly reagents plus rabbit complement before being mixed with the immune cells in the adoptive transfer.

† 24-hr increase in ear swelling (measured in units of 10^{-3} cm) after painting with the specific contactant was measured. Results are expressed as percent of control (immune cells mixed without any suppressor cells). The numbers in parentheses are the results of individual experiments.

Two major points are made by the data. One is that the previously described suppressive regimen does not require that both immunization procedures be done in the same mouse, even though both types of immunization are necessary for suppression to be seen. The second is that the two forms of immunization activate cells with different Ly phenotypes. Comparison of group 3 with group 4 shows that the relevant cell from PCl-immunized mice does not express Ly-2, and comparison with group 5 shows that it does express Ly-1 (i.e., it is an Ly-1 T cell). Similarly, comparison of group 3 with group 6 shows that the TNBSA-immune cells express Ly-2 and comparison with group 7 shows that they do not express Ly-1 (i.e., these are Ly-2 cells). Comparison of group 8 and 9 verifies these conclusions. The reason the TNBSA factor made by Ly-1$^-$ cells works less consistently than does the same material made by unfractionated cells is unclear. Perhaps some Ly-1,2 cells are also involved. However, it is clear that Ly-2$^+$, Ly-1-low, or Ly-1$^-$ cells (i.e., Ly-2 cells) are capable of producing the entire activity found in the TNBSA-immune cell population.

Two Separate "Subfactors" that are Made by Different T Cell Sets are Required for Suppression of CS by Soluble Mediators. The results presented in Table 2 come from experiments that were done in a fashion similar to those that yielded the data presented in Table 1. However, in this case the PCl-immune and the TNBSA-immune cells were cultured by themselves for 48 hr and the biological activity in the culture supernatants was looked for. In these studies all cells were fractionated according to their Ly phenotype. Supernatants from cultures in which only one of the two types of immune cells were present were without significant biological activity (groups 2, 3, and 4). However, mixtures of the supernatants of the PCl-immune Ly-1 cells and the TNBSA-immune Ly-2 cells had significant suppressive activity (group 5). Mixing of the Ly-1 and Ly-2 cells from TNBSA-immune mice did not substantially enhance the ability of these supernatants to interact with those of

Table 2. Cellular requirement for producing factors that suppress the adoptive transfer of CS to normal mice

Group	TNP-immune cells incubated with supernatants from cell cultures of mice immunized with* PCl	TNBSA	CS response in adoptive recipients†
1	–	–	7.0 ± 1.3
2	Ly-1	–	7.5 ± 1.4
3	–	Ly-1	5.8 ± 1.4
4	–	Ly-1 + Ly-2	6.6 ± 1.7
5	Ly-1	Ly-2	2.6 ± 0.3
6	Ly-1	Ly-1 + Ly-2	1.9 ± 0.8

* Cells from immunized mice (see footnote * in Table 1) were fractionated into Ly sets and cultured for 48 hr, and the culture supernatants were mixed (or not), incubated with TNP-immune cells for 45 min at 37°C (2 ml of supernatant per 10^7 cells), and then transferred to normal recipients.

† The 24-hr increase in ear swelling (units of 10^{-3} cm) after painting with the specific contactant. The nonspecific swelling (that of mice that did not receive immune cells) is subtracted, so the results are presented as net increase in swelling, ± SEM.

the PCl-immune cells in reconstituting biological activity (group 6).

The Two Separate Subfactors Show Antigen Specificity in their Ability to Interact with Each Other to Produce Suppressive Activity. We also asked whether either of the two subfactors described had antigen specificity. To do this we immunized some mice by painting their skin with the reactive hapten Ox *in lieu* of PCl, and replaced the TNBSA immunization by Ox conjugated to mouse erythrocytes. In all cases the cells that were to make the Ly-1 subfactor and the cells that were to make the Ly-2 subfactor were cultured separately and the various supernatants were harvested and mixed together. Anti-Ly treatments of these cells were not done.

The results in Table 3 show that: (*i*) the two subfactors must come from cells immunized with the same hapten (TNP or Ox) to produce their suppressive effect, and (*ii*) the finding of subfactors produced by PCl and TNBSA immunization can be extrapolated to the Ox system.

Only One of the Two Subfactors (the Product of the PCl-Immune Ly-1 Cell) Binds Antigen. We next asked if either or both of the subfactors bound to antigen. To do this we made TNP-BGG-Sepharose columns and passed the individual factors through the columns. Elution was performed with excess hapten. Table 4 shows that the eluate from the column over which the product of the cells immunized with PCl was passed could *not* produce suppressive activity when mixed with the eluate from the TNBSA-immune cellular product (group 5), but could when mixed with the effluent (group 6), whereas the PCl effluent would *not* work with the TNBSA effluent (group 7). Thus, the PCl subfactor bound to antigen whereas the TNBSA subfactor did not.

Table 3. Specificity of the suppressive subunit produced by the Ly-2 T cells of mice immunized with TNBSA

Group	Source of Ly-1 factor*	Source of Ly-2 factor†	CS response in adoptive recipients, % of control‡ PCl	Ox
1	PCl	TNBSA	29 (44, 6)	99 (90, 108)
2	Ox	Ox-MRBC	98 (112, 84)	27 (28, 26)
3	Ox	TNBSA	92 (100, 84)	105 (104, 106)
4	PCl	Ox-MRBC	90 (80, 100)	106 (100, 112)

* The 48-hr supernatants from cultures of cells from mice whose skins were painted either with PCl or Ox 24 hr before culture.

† The 48-hr supernatants from cultures of cells from mice immunized intravenously with either TNBSA or mouse erythrocytes (MRBC) conjugated with Ox on days 0 and 4 before harvesting for culture on day 8.

‡ See footnote † in Table 1.

DISCUSSION

The principal finding we have presented is that two molecules (subfactors) coming from cells that express distinct cell surface phenotypes are required for a particular form of antigen-specific suppressive activity. Although the precise molecular mechanism by which these two subfactors work to produce a complete factor is totally unknown, the establishment of such a finding is important for analyses of how antigen-specific biologically active materials are made and how they produce their specific activity.

We have started analyzing the chemical nature of the two macromolecules reported above. The antigen-specific molecule made by the Ly-1 cell is easy to affinity-purify on a hapten immunoabsorbent. Additionally, we have made a heteroantiserum in a rabbit that recognizes this molecule. Preliminary analysis by sodium dodecyl sulfate/polyacrylamide gel electrophoresis suggests that this molecule has a molecular weight of 68,000. The material made by the TNBSA-immunized Ly-2 cell has been harder to purify because it does not bind to antigen. However, we have made a monoclonal antibody that recognizes this product. Interestingly, this product also has an approximate molecular weight of 68,000.

The simplest postulate as to the mechanism by which these two subfactors might work together in an antigen-specific way is that they combine and act in combination as a single unit with two functions. However, our attempts to demonstrate combination, using both solid-phase and fluid-phase conditions, have failed to reveal any evidence that the two subfactors associate with one another. We have also been unable to demonstrate any genetic restrictions in the ability of the subfactors to interact with one another. However, one must interpret this type of negative evidence quite cautiously.

However, assuming that our preliminary results are correct and that the chains do not combine, the question that arises is: "How does the subunit that binds neither to antigen nor to its antigen-binding subfactor impart an antigen-specific interactive event?" The simplest answer to this question would be that the two haptens that we have studied (TNP and Ox) produce different classes of subfactors and that what seems to be an antigen-

Table 4. Antigen-binding capacities of TNP TsF subfactors

Group	TNP-immune cells incubated with supernatants of various cell cultures that were fractionated on TNP-BGG-Sepharose columns PCl* Effluent	PCl* Eluate	TNBSA* Effluent	TNBSA* Eluate	CS response in adoptive recipients†
1	None		None		7.0 ± 1.8
2	+	+	–	–	8.0 ± 1.8
3	–	–	+	+	6.8 ± 1.7
4	+	+	+	+	1.4 ± 0.8
5	–	+	–	+	7.2 ± 1.9
6	–	+	+	–	1.5 ± 0.2
7	+	–	+	–	7.1 ± 1.5

* Supernatants came from cells of mice immunized as described in footnote *, Table 1.

† See footnote † in Table 2.

specific interaction is simply due to functional differences of the subunits and has nothing to do with antigen *per se*. This interpretation is unsatisfying because there is no reason to suspect that the two "complete" factors do not act in a similar way to one another.

Thus, if the apparent antigen specificity is due to a real specificity, it would seem that one must postulate that the suppressive interaction is given a specificity by a third molecule that sees something on the antigen-specific subfactor and something on the nonspecific one, and that in so doing produces a complete antigen-specific factor made up of three separate macromolecules or subfactors. This third hypothetical macromolecule could be made by the assay cells with which the other two subfactors are mixed or by the host into which the assay cells are inoculated. Specificity could be achieved if the hypothesized third subfactor saw antigen and formed an antigen bridge with the antigen-binding molecule, and saw something akin to idiotype (or anti-idiotype) on the non-antigen-binding molecule (the reason for postulating idiotype or anti-idiotype is that this is the second mechanism known to immunologists that can give antigen specificity). In a preliminary test of this notion we have depleted the assay population of Ly-2 cells (note: it is the Ly-1 cell in the assay population that transfers the adoptive immunity) and have found under those circumstances the complete factor is no longer suppressive. This evidence suggests that a third macromolecule is indeed required for biological activity.

The fact that we have been unable to find any major histocompatibility (MHC) determinants on either of the two subunits we are studying is surprising. If one wished to make the generalization, for which no previous exceptions have been described, that all antigen-specific immunoregulatory factors express MHC determinants, one could postulate that the proposed third macromolecule, which is involved in bringing the two macromolecules we have described together, does express an MHC determinant, and in particular I-J.

Another question one might address is: "Why, when both forms of immunization are performed in a single mouse, can one obtain a complete factor in which both macromolecules can be isolated on an antigen-specific affinity column (14)?" One possible explanation is that the association we have found using complete factors from individual mice is artifactual, stemming from the highly reactive nature of the chemical compounds we have used to immunize the donor mice. Thus, it is quite possible that some of the material induced by the TNBSA actually has TNP determinants on it. This subfactor may associate with the subfactor produced by the PCl-immunized Ly-1 cells simply due to the presence of TNP on the TNBSA-induced subfactor. We have been able to produce this type of artifactual binding experimentally, and we tentatively conclude that this accounts for the association found. Of course one must consider the possibility that the hypothetical third subfactor is produced by mice immunized with both PCl and TNBSA. However, if this were the case one would have to postulate the need for four subfactors because the complete TsF made by doubly immunized mice still needs an Ly-2 helper in the assay population to work effectively.

In sum, we have shown that the biological activity of soluble products produced by various T cell subsets depends on a complex interaction between at least two and probably three macromolecules for biological activity to be seen. Many postulates could be put forth for the functional role of each of the separate macromolecules. Functions that may be needed for the biological event to occur include: (*i*) an antigen-bridging reaction with cell surface receptors, (*ii*) a joining of macromolecules into functional units, and (*iii*) delivery of the biologically active peptide to the appropriate receptor on the target cell. At the present time the data are inadequate for rational speculation as to which macromolecule is performing which function, except of course for the antigen-specific one, which we suspect is involved in forming an antigen bridge with the acceptor cell. However, because there is such a high level of complexity in the interactions between the cells responsible for immunoregulation, it is useful and important in analyzing these cellular interactions to consider that the molecular interactions may have similar levels of complexity.

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