SPONTANEOUS RELEASE OF T-CELL RECEPTORS FOR ALLOANTIGENS

I. RECOGNITION OF ALLOANTIGENS AND RECEPTOR RELEASE DYNAMICS

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There is increasing evidence that the membrane of lymphocytes is the organelle with which receptors for antigens have to be associated (1, 2). Much attention has been given to understand the mechanisms of synthesis and release of receptors and considerable progress has been made for receptors from bone marrow-derived (B) lymphocytes (2). For those of thymus-derived (T) lymphocytes, it is generally held that they are difficult to study (2-6). A major problem appears to be the lack of sufficient density of T-cell receptors, as opposed to B-cell receptors. The reason for this could, however, be that T cells are very dynamic cells, shedding receptors at an unexpected speed, and might thus display on their membranes only extremely few receptors.

The results of this communication indicate that this concept might be real, at least as far as T-cell receptors for alloantigens are concerned. T cells appear to release receptors or recognition structures (RS) for \( H-2 \) antigens spontaneously, surprisingly fast, in an orderly manner, and as a result of a temperature-dependent process.

The demonstration of released T-cell receptors for alloantigens has been made possible with an assay system measuring a product of antigenic recognition (PAR). It has recently become apparent that the in vitro interaction of immunocompetent lymphocytes with \( H-2 \) antigens was, in this system, an exclusive property of T cells (7). The present work will show recognition experiments with whole, viable cells and with recognition structures released from these cells.

Materials and Methods

Mice.—Adult animals of inbred mouse strains C57BL/6 and BALB/c, hybrids (C57BL/6 \( \times \) CBA)\( F_1 \) and (BALB/c \( \times \) C57BL/6)\( F_1 \) as well as nude mice, backcrossed to BALB/c mice for

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1 Abbreviations used in this paper: PAR, product of antigenic recognition; PMN, polymorphonuclear; RS, recognition structures; TCM, tissue culture medium 199.

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nine generations (GL. Bomholtgard, Ry, Denmark) were used to obtain suspensions of lymphoid cells.

**Cell Cultures.**—Dissociated suspensions of lymphoid cells (generally from spleens) were counted and adjusted to $2 \times 10^6$/ml cells and were cultivated for varying periods of time in 15 x 60 mm Falcon tissue culture dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in a humid atmosphere of 95% air to 5% CO$_2$. The medium was tissue culture medium (TCM) 199 (Difco Laboratories, Detroit, Mich.) supplemented with 100 IU of penicillin and 50 $\mu$g/ml of streptomycin and fortified with 10% fetal calf serum. To cultivate $10^6$ cells, 0.5 ml of $2 \times 10^6$/ml cells was added to 4.5 ml of medium; to cultivate $2 \times 10^6$ cells (for control purposes) 1 ml of $2 \times 10^6$/ml was added to 4 ml of medium. Cell cultures were terminated by harvesting culture supernates which were centrifuged for 10 min at 850 g. Cell-free supernates were used as the equivalent of $10^8$ or of $2 \times 10^8$ viable cells in the PAR assay.

**PAR Assays.**—The standard procedure for the PAR assay has been described in detail (8, 9) but minor modifications have been made. In brief, rather than cultivating $10^7$ parental strain spleen cells with $10^7$ F1 spleen cells, only $10^8$ cells of each partner were used. 0.5 ml of $2 \times 10^6$/ml parental cells and 0.5 ml of $2 \times 10^6$/ml F1 cells were added to 4 ml of medium in 15 x 60 mm Falcon tissue culture dishes. For control purposes, syngeneic cells were cultured in twice the concentration of cells employed in mixes. Mixed and unmixed spleen cells were cultivated at 37°C for periods determined by the experiments (from 15 min to 7 h) during which time a product of antigenic recognition (PAR) could be elaborated in mixed cultures. The interaction product was determined quantitatively by its granulotactic property. After termination of incubation, culture fluids were harvested, centrifuged (10 min at 850 g) to remove accidentally harvested cells, and were lyophilized. PAR was reconstituted with 0.06 M phosphate buffer, pH 7.8, and the precipitate formed was washed once or twice with buffer. Reconstitution was with 2-3 ml of buffer (per 5 ml of native culture fluid) and washes were with 1 ml of buffer. Insoluble PAR was finally resuspended in buffer to give a 10-fold concentration: 5 ml of native fluid in 0.5 ml of buffer. This constituted five doses of 0.1 ml, of which four were injected intracutaneously into close-clipped backs of outbred hamsters (16 doses/120-150 g host). At the height of the granulotactic response induced by PAR, i.e. 24 h after inoculations, hamsters were killed and skin reactions were removed. Of the four reactions per given sample three were trypsinized individually. Trypsinization was in 3.3 ml of 0.25% trypsin (Difco) for about 6 min in small glass Petri dishes. PMN nuclei released from skin pieces cut into small fragments were counted under phase contrast as described (8, 9). The responses of syngeneic control cultures were used to correct experimental responses (8), expressed as $10^6$ PMN cells per skin reaction.

To test for spontaneously released receptors, a modified form of the PAR assay has been adopted. To 4.5 ml of cell-free culture supernates (to be checked for released receptors), 0.5 ml of $2 \times 10^6$/ml F1 spleen cells were added. Controls consisted of 5 ml of supernates or of $2 \times 10^6$ F1 spleen cells in 5 ml of culture medium. Again, experimental values were corrected for responses obtained in controls. All further steps of the modified PAR assay were identical to those of the standard form.

**Elimination of T and B Cells from Spleen Cell Suspensions.**—T cells were removed by treatment with anti-DC3H and complement. The AKR anti-DC3H antiserum was prepared according to Cerottini et al. (10) and was employed 1:16. Cell suspensions containing $2 \times 10^7$/ml cells were pelleted and resuspended in antiserum, incubated at 37°C under cell culture conditions and after 15 min, guinea pig complement (B-D Mérieux, Marcy l'Etoile, France) absorbed with 80 mg/ml of agarose (Fluka AG, Buchs, Switzerland) and diluted 1:10 was added. Cells were incubated for another 15 min and were then washed thrice with large volumes of Hank's balanced salt solution with 0.1% bovine serum albumin.

The elimination of B cells from spleen cell suspensions was by means of a rabbit antimouse Ig serum and complement. The antiserum was raised by first injecting rabbits intramuscularly with 2 mg of mouse gamma globulin, Fraction II (Miles Laboratories, Inc., Kankakee, Ill.) in Freund's complete adjuvant (Difco). Injections were repeated in about 3-wk intervals, but
antigen was admixed to incomplete adjuvant. Rabbits were bled from the ear about 2 mo after start of immunization and from then on in 2-mo intervals. Serum pools were heat-inactivated and sterilized by filtration. Before treatment of cells, one volume of the antimouse Ig serum was absorbed three times each with 1 vol of packed mouse erythrocytes. Treatment of spleen cells with antimouse Ig and complement was as described for anti-θ and complement, except that antimouse Ig was used 1:20. After elimination of T and B cells from cell suspensions, the number of viable cells was determined and suspensions were readjusted.

RESULTS

Recognition of Alloantigens by T cells and by Spontaneously Released T-Cell Receptors.—When either 10⁶ C57BL/6 spleen cells or 24-h supernate of an equal number of these cells were allowed to recognize CBA alloantigens provided by 10⁶ (C57BL/6 X CBA)F₁ spleen cells, the results shown in Fig. 1 were obtained.

![Graph](image_url)

**Fig. 1.** Recognition of CBA alloantigens on 10⁶ (C57BL/6 X CBA)F₁ spleen cells by 10⁶ C57BL/6 spleen cells (——) or by supernates harvested after cultivation for 24 h of 10⁶ C57BL/6 spleen cells (—). Untreated C57BL/6 cells (●●●●), or supernate (●●●●); C57BL/6 cells treated with rabbit antimouse Ig serum and complement (■■■■), or supernate (■■■■); C57BL/6 cells treated with anti-θ and complement (○○○○), or supernate (○○○○). Syngeneic controls: supernate of 10⁶ C57BL/6 spleen cells + 10⁶ C57BL/6 spleen cells (ΔΔΔΔ), supernate of 10⁶ (C57BL/6 X CBA)F₁ spleen cells + 10⁶ (C57BL/6 X CBA)F₁ spleen cells (▲▲▲▲).
Untreated cells or cells depleted of B cells recognized CBA alloantigens, and when measured for 7 h, formation of PAR followed patterns described previously, including the formation of PAR inhibitor (11). PAR formation was lacking, however, when T-cell-depleted C57BL/6 spleen cells were confronted with alloantigens, confirming earlier results (7) on the T-cell dependency of alloantigenic recognition.

Supernates of 24-h cultures of $10^8$ untreated or similarly treated C57BL/6 spleen cells followed exactly the same patterns. Supernates of untreated or anti-Ig and complement-treated cells recognized CBA antigens surprisingly well, as evidenced by PAR formation. Supernates of anti-$\theta$ and complement-treated cells failed to recognize. No PAR was elaborated when culture supernates were confronted with syngeneic targets, confirming the immunological specificity of the reaction.

Similar experiments using spleen cells from BALB/c or from nude mice and supernates taken from 24-h cultures of these cells are shown in Fig. 2. T-cell receptors associated with BALB/c spleen cells or released from them during in vitro cultivation recognized C57BL/6 antigens of (BALB/c × C57BL/6)F1 spleen cells. Spleen cells from nude mice and 24-h supernates from these cells completely failed to do so.

Previous experiments (7) had shown that, in contrast to spleen cells, bone marrow cells had a considerably decreased capacity to recognize alloantigens, while native thymus cells appeared to have none. These results were thought to reflect proportions of T cells present in these cell suspensions. If the recognition potential of 24-h supernates of parental cells was indeed due to released T-cell recognition structures, a similar pattern should be obtained in comparing 24-h supernates of spleen cells with those of bone marrow or thymus cells. The results of these experiments are shown in Fig. 3. While the response of spleen cells or of spleen cell supernates was similar to that shown in Fig. 1, bone marrow cells or supernates of bone marrow cells revealed considerably smaller activity. Native thymus cells and their supernates showed none.

**Dynamics of Spontaneous T-Cell Receptor Release.**—The results summarized in Figs. 1–3 appeared to indicate that recognition of alloantigens was considerably stronger with cell-free receptors as compared to cell-bound receptors. A possible reason for this quantitative difference could be that within a period of 24 h cultivated spleen cells might shed more than one “generation” of receptors, a process resulting in their accumulation in culture supernates.

To test the dynamics of receptor release, $10^6$ C57BL/6 spleen cells were cultivated and cell-free supernates were obtained hourly for 34 h. Supernates were confronted with $10^6$ (C57BL/6 × CBA)F1 spleen cells and recognition of CBA antigens was allowed to proceed for 2 h. The results are shown in Fig. 4. They appear to indicate that T-cell receptor release was discontinuous with waves of about 8 h. The overall increase in responsiveness to antigen confirmed that,
during the period tested, prolonged cultivation led to an accumulation of receptors.

The peaks and valleys revealed in receptor release dynamics indicated that a given cell culture might at certain periods of cultivation contain more receptors in a functional form than at others. Although pronounced quantitative differences were observed in three independent experiments, it might be argued that the receptor release response as shown in Fig. 4 was a chance observation, possibly due to the fact that receptors and alloantigens were interacting for only 2 h. Release dynamics was therefore tested in a different way, which included confrontation of receptors with alloantigens for 1–7 h. Cultures of C57BL/6 spleen cells were initiated and supernates were harvested after cultivation
Fig. 3. Recognition of CBA alloantigens on $10^6$ (C57BL/6 × CBA)F1 spleen cells by $10^6$ C57BL/6 cells (---) or by 24 h supernate of $10^6$ C57BL/6 cells (----). Spleen cells (●—●) or supernate (●—●); bone marrow cells (○—○) or supernate (○—○); native thymus cells (■—■) or supernate (■—■).

periods which appeared to contain "high" or "low" numbers of receptors (Fig. 4) as evidenced by higher or lower formation of PAR. These supernates were mixed with (C57BL/6 × CBA)F1 spleen cells and recognition of CBA alloantigens was for up to 7 h. The results (not corrected for controls) shown in Fig. 5 confirmed the discontinuous receptor release and also revealed that a 2-h interaction period was representative for both "high" and "low" levels of receptors.

It might be expected that receptor release depended on the numbers of cells cultivated and on temperature. To test these premises, experiments were initiated in which 10-fold dilutions, starting with $10^8$, of C57BL/6 spleen cells were cultivated for 24 h at 37°C and at 4°C. From the results in Fig. 6 it can be seen that spleen cells kept at 4°C did not release receptors, whereas cells cultivated at 37°C did. At the latter temperature, cell concentrations higher than $10^6$ cells per dish resulted in inferior responses, possibly for reasons of crowding. Cell
concentrations lower than $10^5$/dish showed decreased receptor release, most likely a dilution effect.

While the release of T-cell receptors appeared to be temperature-dependent, it might well be that once receptors were released, their interaction with alloantigens was temperature-independent. To test this, recognition of CBA antigens by $10^6$ C57BL/6 spleen cells and by 24-h supernates of $10^6$ C57BL/6 spleen cells was studied at $4^\circ$C and at $37^\circ$C. The results shown in Fig. 7 indicate that

Fig. 4. Receptor release dynamics. Recognition of CBA alloantigens on $10^6$ (C57BL/6 × CBA)F1 spleen cells by supernates harvested after cultivation of $10^6$ C57BL/6 spleen cells for time intervals indicated on the abscissa. PAR was determined after interaction of hourly harvested supernates with CBA antigens for 2 h at $37^\circ$C. Each point represents the mean value of three experiments.
receptors released at 37°C recognized alloantigens both at 4°C and at 37°C. This contrasted with recognition by cells, which occurred only at 37°C, possibly because cell-cell recognition also depended on receptor release. When tested at 37°C, the reaction of released receptors with antigen was extremely fast and complete within 30 min. At 4°C recognition must also have been very fast, a high value being obtained at 1 h, the shortest interval tested. Whereas at 37°C decreasing amounts of PAR would have been expected by 7 h (see e.g. Figs. 1

![Graph showing receptor release dynamics.](image)

**Fig. 5.** Receptor release dynamics. Recognition of CBA antigens on 10^6 (C57BL/6 × CBA)F1 spleen cells by receptors present in supernates of 10^6 C57BL/6 spleen cells harvested after cultivation periods indicated in the Figure. Release maxima, •—•; release minima, O—O.
and 2) levels at 4°C remained high, indicating that no PAR inhibitor (11) was formed at this temperature.

The release dynamics of T-cell receptors (Fig. 4) showed that cells cultivated for 24 h at 37°C were well capable to continue shedding further RS. A confirmation of this and an answer to the question of how spleen cells kept at 4°C for 24 h would respond to alloantigens is given by results summarized in Fig. 8. For these experiments 10^6 C57BL/6 spleen cells were cultivated in Falcon dishes for 24 h at 37°C or at 4°C. After cultivation, supernates were removed and were immediately replaced by fresh medium to which 10^6 (C57BL/6 × CBA)F1 spleen cells were added. Recognition of CBA antigens by cultivated cells was followed for 7 h and was compared to the response induced by freshly prepared C57BL/6 spleen cells. The results show that cells prevented from releasing receptors by being kept at 4°C recognized antigen just as well as freshly prepared cells. Cells cultivated at 37°C showed increased responsiveness, presumably because they were well adapted to the medium and were actively
secreted receptors. As before, 24-h supernates of 37°C cultures of 10⁶ C57BL/6 spleen cells did, whereas those from 4°C cultures did not contain receptors.

Lack of Spontaneous Release of Alloantigens.—Would alloantigens in a form capable of being recognized in the PAR assay be released as easily as T-cell receptors? To test this, 10⁶ spleen cells from (C57BL/6 × CBA)F₁ mice were cultivated for 24 h and culture supernates were tested for released CBA alloantigens by confronting them with 10⁶ C57BL/6 spleen cells or with 24-h culture supernates of 10⁶ C57BL/6 spleen cells. In departure from the usual procedure, the latter samples consisted of 2.5 ml each of parental and F₁ supernate each obtained from 2 × 10⁶ cells/dish. The results are shown in Fig. 9. C57BL/6 spleen cells recognized CBA alloantigens present on (C57BL/6 × CBA)F₁ cells normally, but there was no reaction between C57BL/6 spleen cells or RS-containing supernates of these cells with supernates of F₁ spleen cells, indicating
that alloantigens were not released spontaneously in recognizable form under the culture conditions employed.

**DISCUSSION**

The experiments reported demonstrate that the principle released from cultivated lymphoid cells of normal mice appears to be spontaneously shed receptor or recognition structure (RS) for alloantigens. These receptors were secreted into the surrounding medium by untreated spleen cells, by spleen cells depleted of B cells, but not by spleen cells depleted of T cells nor by spleen cells from nude mice lacking T cells (12-14). Other cell suspensions, like those from bone marrow, containing few T cells (15, 16) or from native thymus, lacking functional T cells (7, 17, 18) shed few or no receptors. Control supernates demonstrated lack of response to syngeneic cells. Thus, secreted receptors appeared to be of T-cell origin.
Qualitatively, released and cell-bound receptors showed similar recognition patterns. These were characterized by high amounts of the recognition product (PAR) during the first few hours, followed by a decrease of the product after prolonged interaction periods. This decrease has been described before (11) and has been attributed to the formation of an inhibitor. While the nature of the inhibitor is still unclear, the use of cell-free receptors as an "aggressor population" has now revealed that it must be formed by F1 target spleen cells, but only at 37°C and not at 4°C (Figs. 1 and 7).

From a quantitative point of view, released receptors were much more potent in recognizing alloantigens than were receptors presented in the form of immunocompetent cells. The reason for this somewhat puzzling observation became apparent when the dynamics of receptor release was studied. This revealed that cultivated cells shed receptors in several waves, a process resulting in receptor accumulation in the medium. While this might be a straightforward explanation for the increased response of supernates harvested from 24-h or more cultures of lymphoid cells, the reasons for the discontinuous receptor release are not yet clear. The 8-hourly release waves observed might represent division cycles of a small population of lymphocytes. Periods characterized by
bursts of receptor release were followed by what appeared to be periods of neutralization of released receptors, as marked by an actual decrease of the number of functional receptors. At present it can only be speculated that receptor release is, like all biological processes, somehow controlled. Preliminary observations have so far revealed that the release dynamics can be characterized by even more dramatic amplitudes, depending on the recognition system studied. The heightened response observed with free receptors as compared with receptors still “bound” to aggressor cells at the initiation of the recognition test might chiefly be due to quantitative reasons. Evidence of an indirect nature (Ramseier and Lindenmann, to be published) has indicated that PAR might be an antibody-antigen complex with T-cell receptors acting as antibody. Thus, provided sufficient antigen is present, more PAR will be formed the greater the number of free receptors in the recognition system. Furthermore, lack of receptor release at 4°C (Fig. 6) and lack of PAR formation by mixed spleen cell cultures at 4°C (Fig. 7) indicated that in mixed cell cultures receptors first have to be shed before reactivity with antigen can take place. Although comparisons on recognition kinetics appeared to indicate that antigen-induced receptor release (Figs. 1–3) is faster than receptor secretion in the absence of antigen (Fig. 4), it is nevertheless a process requiring some time (2 h in the system studied). Consequently, PAR formation with already released receptors is also faster.

Receptor release seems to require metabolic activity. After cultivation for 24 h at 4°C no recognition structures could be detected in the surrounding medium. The capacity to form receptors, however, had not been lost, since these cells were perfectly capable of recognizing alloantigens when confronted to them at 37°C. In contrast to the release process, the interaction of already released receptors with antigen was temperature-independent. Since lack of receptor release at low temperatures paralleled lack of recognition, one might conclude that unresponsiveness at 4°C was solely due to prevented release. It appears, therefore, that recognition by parental cells of alloantigens present on F1 cells as revealed in the PAR assay does not depend primarily on cell-cell contact, but on whether or not parental aggressors can release their T-cell receptors.

Whatever the mechanisms of receptor release and its control may be, it was surprising to find the release so regular and so fast. This indicated that receptor synthesis and secretion might be of an unexpected speed. Should this be true, then the situation of T-cell receptors resembles much more that of immunoglobulins shed by plasma cells, and contrasts markedly with that of B-cell receptors. Compared to the latter, T-cell receptors and immunoglobulins secreted by plasma cells are subject of rapid turnover. The difficulties in demonstrating surface immunoglobulins on plasma cells (19, 20) despite the fact that these cells are actively producing large amounts of Ig, might therefore apply equally well to T cells. At any given time T cells might display only very few receptors on their surface membrane. The present experiments indicate that this is most probably not a sign of receptor scarcity but of active secretion. Rapid
turnover of surface constituents by T cells is also indicated by the work of Wernet et al. (21) showing that lymphocytes whose receptors had presumably been shed recovered rapidly their reactivity within 7–24 h of cultivation.

The results presented here contrast with many careful studies on T-cell receptors. In most attempts, these receptors have successfully escaped detection by methods applicable in revealing B-cell receptors, such as immunofluorescence (22, 23), radioactively labeled antibodies (22, 24), and radiiodination of cell surface proteins (3, 4). With this latter method, however, Marchalonis et al. (25) and Cone et al. (26) reported successful demonstration of IgM receptor molecules on T cells, possibly buried deeply in the cell membrane and thus inaccessible to antibodies employed to reveal them. In a very careful study, Lisowska-Bernstein et al. (27) using surface labeling techniques and biosynthetic labeling could not confirm these findings and emphasized the possibility of artifacts inherent in the methodology. Since contrary to perhaps most expectations T-cell receptors are released spontaneously, studies on their role in interactions with B cells and on their immunochemical nature should be facilitated.

Sharply in contrast to the surprising ease by which T-cell receptors appear to be released, a spontaneous release of alloantigens recognizable in the PAR assay could not be observed under the culture conditions employed. Although this result is based only on a 24-h cultivation period, it is consistent with findings by Vitetta and Uhr (2) and agrees with the interpretation that H-2 antigens are constitutive proteins of the cell membrane.

SUMMARY

In vitro cultivation of murine spleen cells resulted in a spontaneous release of receptors for alloantigens. This was revealed by the capacity of cell-free culture supernates to recognize alloantigens as measured in the PAR assay. Qualitatively, recognition responses obtained with these supernates reproduced faithfully those found with the corresponding cells.

Large amounts of receptors were released by untreated spleen cells and by spleen cells treated with a rabbit antimouse immunoglobulin serum and complement, smaller amounts were released by bone marrow cells, and native thymus cells released none. Spleen cells from nude mice and spleen cells from normal mice treated with anti-θ serum and complement showed no release of receptors. It was concluded that receptors active in the PAR test were of T-cell origin.

Release of T-cell receptors was found to be discontinuous and proceeded in waves. The amount of released receptors depended on the number of cells cultivated. Release occurred at 37°C but not at 4°C. Interaction with antigen, however, was temperature-independent. In contrast to T-cell receptors, a release of H-2 antigens could not be detected with the culture conditions employed.
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