CELL-BOUND RECEPTORS FOR ALLOANTIGENS ON NORMAL LYMPHOCYTES

II. ANTIALLOANTIBODY SERUM CONTAINS SPECIFIC FACTORS REACTING WITH RELEVANT IMMUNOCOMPETENT T LYMPHOCYTES*

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Antibody molecules, besides being able to bind relevant antigenic determinants, can also function as immunogens. Antiantibodies can thus be produced against various parts of an antibody molecule. Antibodies directed at the antigen-binding sites or other unique parts of the variable area of the antibody molecule are called anti-idiotypic antibodies (1). Such anti-idiotypic antibodies are regularly produced by F1 hybrid animals injected with alloantibodies raised in one parental strain against the other parent (2, 3). Proof that it is the F1 hybrid which elaborates such antialloantibodies comes from an analysis of their allotype characteristics (4), the kinetics of their synthesis (5), and the fact that they are induced by determinants on the Fab portion of alloantibodies (6).

Originally, antialloantibodies were detected by their effect on recognition of alloantigens by immunocompetent cells, which was specifically inhibited in certain in vitro systems (2), although not in others (7). Tests developed later included binding of radiolabeled antialloantibody (3) and gel precipitation (8). In vivo, the most striking effect was suppression of graft-vs.-host (GvH)† reactivity of parental cells in F1 hybrids by passive transfer of antialloantiserum (9). This suggested that the immunocompetent parental T lymphocyte, generally regarded as the principal actor in GvH, carried idiotypic determinants. It could indeed be shown that unfractionated parental lymphoid cells bound radiolabeled antialloantibody (3).

Using radiolabeled antialloantibodies, separation procedures to obtain "pure"...
732

CELL RECEPTORS FOR ALLOANTIGENS. II

B and T lymphocytes, and allogeneic monolayers as specific immunoadsorbants, we found that antialloantibodies would only bind in significant quantities to B lymphocytes (10). Furthermore, all idioype-positive B lymphocytes had similar antigen-binding specificity as evidenced by their adsorption to the relevant, allogeneic fibroblast monolayer. Preincubation with antialloantibody specifically blocked binding of B lymphocytes to monolayers. These results thus failed to demonstrate idioypic receptors on T lymphocytes as measured by the direct radioimmunoassay. However, in preliminary tests the antialloantibody-containing sera selectively inhibited binding of immunopotent T cells to the relevant monolayers as tested by subsequent GvH reactions. In the present experiments we have analyzed the capacity of antialloantibody-containing sera to affect immunocompetent T lymphocytes, and have established specific inhibitory effects on purified T lymphocytes of relevant genotype.

Materials and Methods

Animals, alloantisera, antialloantisera, iodination, and absorption of antialloantibodies, rabbit antirat Ig and anti-T sera, normal lymphoid cell suspensions, reactions between iodinated antialloanfibodies and lymphoid cells, cytotoxic assays, column purifications of B and T cells, rat fibroblast monolayers, and treatment of lymphoid cells with antisera were as described in the preceding paper (10).

Incubation of Lymphoid Cells with Rat Fibroblast Monolayers.—$10^8$ spleen cells pretreated in the manners described in the text, suspended in tissue culture medium (TCM) 199 supplemented with 15% fetal calf serum (FCS) and antibiotics, were left in contact with rat fibroblast monolayers for 180 min at 37°C, shaking very gently after 90 min. After incubation culture flasks were shaken gently for 15 min on a rocker. Cells in the supernate were harvested and the attached cells were eluted with Eagle's basal medium (BME) containing 0.0025 M EDTA by shaking the flasks again on the rocker. Cells from the supernate and cells eluted with EDTA were then washed and trypsinized with 0.25% trypsin for 10 min at 37°C. Trypsinization was stopped by adding 10% FCS. Cells were washed twice with BME, filtered through nylon mesh, and incubated overnight in TCM 199 with 15% FCS and antibiotics. The next day, cells were washed twice with BME; they were then used for testing their ability to absorb iodinated antialloantibody and their GvH reactivity.

Popliteal Lymph Node Assay.—Usually $5 \times 10^6$ lymphoid cells in 0.1 ml were injected into the footpads of 5-8-wk old F1 animals. Rats were killed 7 days later and the lymph nodes were excised and weighed (9, 11).

RESULTS

Role of T Cells in the Local Popliteal Rat Lymph Node Assay.—The GvH potential of lymphocytes is thought to measure T-lymphocyte activity (12). Since it has been claimed (13) that B lymphocytes may participate in certain GvH reactions we considered it essential to establish that our test system measured exclusively the immune reactivity of T lymphocytes. Comparatively pure T and B lymphocyte populations can be produced from mixtures by several procedures. We used the capacity of anti-T or anti-Ig sera in the presence of complement to selectively lyse T and B lymphocytes, respectively. The lymphocytes thus obtained were then tested for their capacity to produce a local popliteal
lymph node reaction (Table I). Treatment with anti-T serum almost completely wiped out GvH reactivity whereas treatment with anti-Ig serum had no such effect. We concluded that only T lymphocytes were important in this assay.

Capacity of Antialloantibody-Containing Serum to Inhibit the GvH Reaction of Purified Parental T Lymphocytes.—We had shown previously that antialloantibody serum produced in F1 hybrids by the injection of alloantibodies from one parental strain against the other specifically suppressed the GvH reactivity of lymphocytes from the former parental strain (9). The lymphocytes tested contained both T and B cells. The results in Table I suggest that inhibition involved T lymphocytes. However, the possibility remained that the antialloantibodies might primarily affect B lymphocytes; reaction with the idiotype-bearing cells might then create antigen-antibody complexes which inhibited the GvH reaction. Therefore, we tested highly purified T lymphocytes from both parental strains for their ability to provoke a GvH reaction in animals actively immunized with alloantisera (Table II). Even with “pure” T lymphocytes there was a selective, significant inhibition of the GvH reaction. From the data in Tables I and II we concluded that the inhibitory activity of antialloantibody serum as measured in the local popliteal lymph node assay was due to a direct inhibition of T lymphocytes.

Capacity of Antialloantibody-Containing Sera to Block the Binding of Specific T Lymphocytes to the Relevant Monolayers.—Little doubt exists that immune T lymphocytes involved in cell-mediated killing can be removed specifically by adsorption onto target monolayers. However, with normal T lymphocytes the situation is less clear. It has been reported that normal T lymphocytes potentially reactive in GvH or as killer cells can be removed by incubation on appropriate monolayers (14–16). Others have failed to remove normal T lymphocyte activity by such procedures (17). Whatever the reason for these conflicting
TABLE II

GvH Induced by Purified Parental T Lymphocytes. Inhibition by Immunization of (Lewis × DA) F₁ Hosts with Parental Alloantibodies

| Hosts immunized with DA anti-Lewis* | DA node mg | Lewis node mg | Ratio |
|------------------------------------|------------|---------------|-------|
|                                    | 16.0       | 53.5          | 0.30  |
|                                    | 38.0       | 102.7         | 0.37  |
|                                    | 44.5       | 111.5         | 0.40  |
|                                    | 56.8       | 116.1         | 0.49  |
|                                    | 34.6       | 65.5          | 0.53  |
|                                    | 43.7       | 83.0          | 0.53  |
|                                    | 41.4       | 70.2          | 0.59  |
|                                    | 48.5       | 75.8          | 0.64  |
|                                    | 56.7       | 80.6          | 0.70  |
|                                    | 37.5       | 51.2          | 0.73  |
|                                    | 39.8       | 51.8          | 0.77  |
|                                    | 45.0       | 38.5          | 1.17  |

Mean log ratio ± SE = -0.240 ± 0.044§

| Hosts immunized with Lewis Anti-DA* | DA node mg | Lewis node mg | Ratio |
|-------------------------------------|------------|---------------|-------|
|                                     | 92.5       | 62.4          | 1.48  |
|                                     | 99.0       | 60.0          | 1.65  |
|                                     | 97.3       | 57.7          | 1.69  |
|                                     | 126.8      | 65.0          | 1.86  |
|                                     | 133.4      | 64.9          | 2.06  |
|                                     | 49.0       | 23.4          | 2.09  |
|                                     | 98.2       | 43.9          | 2.24  |
|                                     | 88.7       | 38.4          | 2.31  |
|                                     | 127.0      | 51.3          | 2.48  |
|                                     | 71.2       | 23.4          | 3.04  |

Mean log ratio ± SE = 0.314 ± 0.028§

* F₁ hosts were immunized by two subcutaneous injections of 1 ml of alloantiserum at 10-day intervals. GvH reactions were induced two days after the second injection by purified parental T lymphocytes. DA cells were injected into one footpad and Lewis cells into the other.

† Popliteal lymph node weights (mg), in individual rats. "Lewis node" refers to the node from the side on which Lewis T cells were injected, "DA node" to the side of the DA T cell.

§ Both values differ significantly from 0.

results, we have found it possible to remove rat GvH-reactive cells by preincubation on the relevant monolayer. Table III shows a comparative assay when normal parental lymphocytes (both B and T lymphocytes) were allowed first to react with syngeneic or allogeneic fibroblast monolayers. The incubation was
TABLE III
Interference with the Adsorption of GvH-Reactive Cells and $[^{125}I]$Antialloantibody-Reactive Cells onto Allogeneic Fibroblast Monolayers. Effect of Anti-Ig and Antialloantibody Sera

| Treatment with sera | Cells remaining in the supernatant after adsorption onto: |
|---------------------|-----------------------------------------------|
|                     | DA monolayer | Lewis monolayer | Mean log ratio ± SE* |
|                     | GvH activity | Binding of $[^{125}I]$anti-(L anti-DA) | GvH activity | Binding of $[^{125}I]$anti-(DA anti-L) |
| mg | cpm | mg | cpm |
|---|---|---|---|
| DA | F1 anti-(DA anti-L) | 75.2 | ND | 65.3 | 316 | 0.063 ± 0.060 |
| | F1 normal serum | 88.8 | ND | 58.4 | 127 | -0.188 ± 0.025 |
| | Rabbit antirat Ig | 94.2 | ND | 62.8 | 369§ | -0.178 ± 0.029 |
| Lewis | F1 anti-(L anti-DA) | 85.5 | 486 | 72.1 | ND | 0.069 ± 0.047 |
| | F1 normal serum | 77.6 | 110 | 99.3 | ND | 0.115 ± 0.164 |
| | Rabbit antirat Ig | 66.7 | 630§ | 107.5 | ND | 0.198 ± 0.045 |

* Ratio of lymph node weights induced by cells after absorption with Lewis (L) monolayers over lymph node weights induced by cells after absorption with DA monolayers. Smallest significant value ($P < 0.05$) is 0.110. Values not significantly different from zero (boldfaced) indicate that allogeneic monolayer was not more effective in removing GvH-reactive cells than syngeneic monolayer.

† Mean weight of three lymph nodes (mg). Boldfaced values indicate reduced absorption by relevant monolayer.

§ Mean uptake of radioactivity by $5 \times 10^6$ cells (cpm). Boldfaced values indicate reduced absorption by relevant monolayer.

Carried out in the presence of anti-Ig serum, normal F1 serum or antialloantibody-containing serum of relevant specificity. The capacity of the reagents to interfere with B-cell binding was analyzed by radioimmunoassay (3) and with T-cell binding by the GvH test. Both anti-Ig and antialloantibody serum specifically inhibited the binding of B cells to the relevant monolayer, whereas only the antialloantibody serum would block the binding of T lymphocytes (underlined figures in Table III). Thus, anti-(DA anti-Lewis) serum inhibited the attachment of DA cells onto Lewis monolayers, as evidenced by greater GvH reactivity of the cells remaining in the supernate and by the fact that such serum-treated cells were no longer preferentially absorbed by the allogeneic monolayer as compared to the syngeneic monolayer (Table III, line 1). In contrast, DA cells treated with normal F1 serum were depleted from the supernate after contact with the Lewis monolayer, whereas the syngeneic monolayer had no such effect (Table III, line 2). Absorption of GvH-reactive cells by the allogeneic monolayer was not impaired by pretreatment with antiserum to rat Ig (Table III, line 3). However, both antialloantibody and anti-Ig inhibited the absorption by the allogeneic monolayer, of those cells which were capable of binding significant amounts of iodinated antialloantibody, confirming the B-cell nature...
of these cells (Table III, lines 1 and 3). Corresponding results were obtained with Lewis cells as aggressors (Table III, lines 4–6).

In a further series of tests we analyzed the effects of anti-Ig, anti-T, normal F1, or antialloantibody sera on the capacity of GvH-reactive cells to bind to syngeneic or allogeneic monolayers. We have already shown that B-cell binding to monolayers is inhibited by anti-Ig and antialloantibody sera (10 and Table III). When GvH-reactive cells were incubated on syngeneic monolayers (Table IV) the presence of various antisera did not affect the distribution of reactive

### TABLE IV

**Binding and Elution of GvH-Reactive Cells to Cellular Monolayers. Interference by Anti-Ig, Anti-T, and Antialloantibody Sera**

| Splenocytes Serum treatment of spleen cells | Monolayers | GvH reactivity of: | Cells in supernate* | Eluted cells* | Mean log ratio ± SEI |
|--------------------------------------------|------------|---------------------|---------------------|---------------|---------------------|
| Lewis, Rabbit antirat Ig DA                | 27.3       | 42.5                | −0.192 ± 0.019      |               |                     |
| Lewis, Rabbit anti-T                        | 47.9       | 64.2                | −0.111 ± 0.043      |               |                     |
| (DA X F1) normal                            | 25.4       | 37.8                | −0.185 ± 0.077      |               |                     |
| (DA X F1) anti-(L anti-DA)                        | 30.3       | 33.3                | 0.297 ± 0.018       |               |                     |
| Lewis, Rabbit antirat Ig Lewis               | 39.3       | 36.3                | −0.035 ± 0.014      |               |                     |
| Lewis, Rabbit anti-T                        | 24.6       | 32.5                | 0.033 ± 0.014       |               |                     |
| (DA X F1) anti-(L anti-DA)                        | 37.5       | 37.1                | 0.007 ± 0.039       |               |                     |
| DA, Rabbit antirat Ig DA                    | 46.9       | 46.7                | −0.006 ± 0.039      |               |                     |
| DA, Rabbit anti-T                            | 50.5       | 53.1                | −0.029 ± 0.069      |               |                     |
| (DA X F1) normal                            | 36.0       | 36.1                | 0.008 ± 0.032       |               |                     |
| (DA X F1) anti-(DA anti-L)                        | 34.9       | 35.1                | 0.036 ± 0.014       |               |                     |
| Lewis, Rabbit antirat Ig Lewis               | 33.9       | 34.2                | −0.151 ± 0.034      |               |                     |
| Lewis, Rabbit anti-T                        | 49.1       | 67.8                | −0.145 ± 0.044      |               |                     |
| (DA X F1) anti-(DA anti-L)                        | 20.6       | 41.3                | −0.304 ± 0.018      |               |                     |
| (DA X F1) anti-(DA anti-L)                        | 42.3       | 15.9                | 0.435 ± 0.060       |               |                     |

* Mean weight of three lymph nodes (mg).

† Ratio of node weight induced by cells in supernate over node weight induced by cells in eluate. Boldfaced values denote blocking of specific adsorption.

From the data in Tables III and IV we conclude that (a) GvH-reactive cells
could be removed by preincubation in vitro with the appropriate monolayers and (b) of the sera tested, those containing antialloantibody could block the binding of both GvH-reactive cells and B lymphocytes, whereas (c) anti-Ig serum would only block the binding of B lymphocytes.

Cytotoxic Effects of Antialloantibody Serum on GvH-Reactive Cells.—We have found previously that antialloantibody sera in the presence of complement were cytotoxic for the relevant, idiootype-positive B lymphocytes (10). We now tested whether antialloantibody-containing serum in the presence of complement was similarly cytotoxic for GvH-reactive lymphocytes. Cells to be tested were incubated with antialloantisem in the presence or absence of complement or with complement alone. They were then washed, trypsinized, allowed to recover, and tested for their capacity to induce GvH reactions in the local popliteal lymph node assay. As controls, anti-Ig or anti-T sera were tested for cytotoxic activity in the same assay. Anti-T or antialloantibody-containing sera inhibited GvH activity in this assay (Table V) whereas, as expected from the previous findings, anti-Ig and complement did not reduce the GvH potential. Table VI shows that antialloantibody-containing serum without complement was ineffective in this

### TABLE V

| Cells injected | Host       | Injected cells treated with | Mean lymph node weights* | Mean log ratio ± SE  |
|----------------|------------|----------------------------|--------------------------|----------------------|
| Lewis          | (L × BN)F₁ | F₁ anti-(L anti-BN) + complement | 22.7 mg | 0.444 ± 0.062† |
|                |            | Complement alone           | 62.1 mg | 0.499 ± 0.062‡ |
|                |            | Rabbit anti-T + complement  | 32.6 mg | 0.003 ± 0.199 |
|                |            | Complement alone           | 56.1 mg | 0.037 ± 0.065 |
|                |            | Rabbit anti-rat Ig + complement | 55.8 mg | 0.037 ± 0.199 |
| BN             | (L × BN)F₁ | F₁ anti-(BN anti-L) + complement | 51.9 mg | 0.003 ± 0.053 |
|                |            | Complement alone           | 95.2 mg | 0.136 ± 0.034† |
|                |            | Rabbit anti-T + complement  | 52.9 mg | 0.003 ± 0.053 |
|                |            | Complement alone           | 81.0 mg | 0.003 ± 0.053 |
|                |            | Rabbit anti-rat Ig + complement | 91.3 mg | 0.003 ± 0.053 |
|                |            | Complement alone           | 76.2 mg | 0.003 ± 0.053 |
| DA             | (DA × BN)F₁| F₁ anti-(DA anti-BN) + complement | 27.5 mg | 0.463 ± 0.180† |
|                |            | Complement alone           | 97.1 mg | 0.003 ± 0.053 |
|                |            | Rabbit anti-T + complement  | 40.4 mg | 0.003 ± 0.053 |
|                |            | Complement alone           | 107.3 mg | 0.003 ± 0.053 |
|                |            | Rabbit anti-rat Ig + complement | 81.2 mg | 0.003 ± 0.053 |
|                |            | Complement alone           | 75.2 mg | 0.003 ± 0.053 |
| BN             | (DA × BN)F₁| F₁ anti-(BN anti-DA) + complement | 42.2 mg | 0.404 ± 0.176† |
|                |            | Complement alone           | 112.0 mg | 0.136 ± 0.034† |

* Mean weight of three nodes (mg).
† Significantly different from 0.
CELL RECEPTORS FOR ALLOANTIGENS. II

**TABLE VI**

GvH Reaction of Parental Lymphoid Cells Treated with Antialloantibodies plus Complement in Comparison to the GvH Reaction of Parental Lymphoid Cells Treated with Antialloantibodies Alone or Complement Alone

| Cells injected | Host | Injected cells treated with | Mean lymph node weights ± SE* | Mean log ratio ± SE |
|----------------|------|-----------------------------|-----------------------------|---------------------|
| DA (DA × BN)/F₁ | Anti-(DA anti-BN) + complement | 17.7 ± 1.0 | -0.327 ± 0.038† | |
|                | Anti-(DA anti-BN)              | 37.8 ± 2.4 | ‡                       | |
|                | Anti-(DA anti-BN) + complement | 18.3 ± 1.1 | -0.209 ± 0.031‡        | |
|                | Complement alone               | 30.5 ± 3.2 | ‡                       | |
| Lewis (L × BN)/F₁ | Anti-(L anti-BN) + complement | 15.7 ± 0.8 | -0.208 ± 0.027†        | |
|                | Anti-(L anti-BN)               | 25.3 ± 1.2 | ‡                       | |
|                | Anti-(L anti-BN) + complement | 13.6 ± 0.9 | -0.238 ± 0.024‡        | |
|                | Complement alone               | 21.5 ± 1.0 | ‡                       | |

* Mean of between 16 and 22 lymph nodes.
† Significantly different from zero.

Assay. This suggested that the inhibition of GvH reactivity was due to killing of the reactive cells rather than to blocking. Cytotoxicity was not apparent by trypan blue uptake, probably because antisera-sensitive cells were infrequent. Not all antialloantibody-containing sera proved cytotoxic for GvH-reactive cells. Of the eight sera positive by other tests only four appeared to be cytotoxic. We would thus conclude that several of our antialloantibody-containing sera behaved as though they contained antibodies cytotoxic for the relevant GvH-reactive T lymphocytes.

**DISCUSSION**

Our present data support the view that antisera produced in F₁ hybrids to the alloantibodies of one parental strain against the other contain antibodies reactive with antigen-binding receptors on T lymphocytes. Such receptors would be present on T lymphocytes from the strain that produced the alloantibodies used for inoculation into the F₁ hybrid animals, and would carry specificity for the alloantigens of the other parental strain. The evidence for this comes from the findings that "pure" T lymphocytes were selectively inhibited in their GvH reactivity when inoculated into F₁ hybrids actively immunized with alloantibodies. Furthermore, binding of GvH-reactive cells (we found as have others that these cells were T lymphocytes) to the relevant allogeneic monolayers was inhibited by antialloantibody-containing sera. And finally, in several experiments we could demonstrate that T lymphocytes incubated with antialloantibody and complement no longer induced GvH reactions. Our data thus confirm and extend previous reports of the specific inhibitory activity of antialloantibody serum on GvH reactivity (8, 9), and demonstrate that the effect occurs at the level of the T lymphocyte.
There are, however, several seemingly contradictory findings in this field. Antialloantibody IgG radiolabeled with $^{125}$I bound significantly to the appropriate parental lymphocytes, but apparently only to B lymphocytes (10). Yet, the same serum (pure IgG has not been tested) inhibited the binding of T lymphocytes to the relevant allogeneic monolayers and inhibited the GvH reactivity of inoculated T lymphocytes in vivo (present article and 9). There are several possible explanations. The T cells might not have receptors with the B-cell idiotype against which the antialloantibodies are directed. Alternatively, the T lymphocytes might have the same receptors as B lymphocytes but fail to express them in a way allowing detection by binding assays. A further possibility is that the specific factor in the antialloantibody-containing sera that reacts with T lymphocytes is not an antialloantibody after all. It might be a complex consisting of alloantibody and antigen of the other parental strain, produced in the F1 hybrid as a consequence of the injection of alloantibody. The strongest argument against this latter possibility would seem to be the demonstration (Tables V and VI) that four of the antialloantibody-containing sera appeared to be cytotoxic for the appropriate T lymphocytes in the presence of complement. It is theoretically conceivable, although unlikely, that antigen-antibody complexes in antigen excess might bind to the relevant antigen-binding T cells, then fix complement via antibody and kill T lymphocytes indirectly.

However, it is possible that the alloantisemur injected into the F1 hybrids to induce antialloantibodies might contain, besides “classical” alloantibody, some soluble antigen-specific lymphocyte product leading to the elaboration of antireceptor antibodies (Ramseier and Lindenmann, to be published). Antireceptor antibodies raised against parental spleen cells have been shown to inhibit lethal GvH (18). When “pure” T-lymphocyte suspensions were used as the immunizing agent, inhibition of local GvH could also be demonstrated (Wigzell and Binz, in preparation). In addition, “pure” T cells proved reactive against foreign alloantigens in the PAR assay (19) and were able to induce formation of antireceptor antibodies (20). It appears even possible to raise xenogeneic sera against T-cell receptors by immunization of rabbits with killer mouse cells sensitized across an $H$-2 barrier; after proper absorption this serum inhibited only the relevant killer cells (21).

In conclusion, the present article shows that sera from F1 hybrids inoculated with posttransplantation alloantibodies produced in one parental strain against the other contain specific factors capable of reacting with B and T lymphocytes of the alloantibody donor genotype. It is unknown whether the same specific factor reacts with both B and T lymphocytes from the same individual. The factor reactive with B lymphocytes is known to be antibody, some of which is IgG (10). We have no evidence about the chemical nature of the anti-T factor. Since it is specific and sometimes cytotoxic for the relevant T lymphocytes it may well be antibody to the idiotype of T receptors. Whatever the exact mechanism for the observed specific T-cell suppression, it holds promises both for theoretical insights and practical applications.
The rat popliteal node graft-vs.-host assay was shown to depend on the presence of parental T cells in the inoculum. Antialloantisera raised in F1 hybrid rats against alloantibodies of one parent directed at transplantation antigens of the other parent displayed some or all of the following specific effects on parental T cells: They inhibited local GvH by purified T-cell suspensions; they blocked the capacity of GvH-reactive cells to adsorb onto fibroblast monolayers of the relevant genotype; together with complement, they killed GvH-reactive cells.

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