SIMULTANEOUS COMPLEMENTARY IDIOTYPIC RESPONSES: ABSENCE OF RECIPROCAL REGULATION*

BY DONALD A. ROWLEY, GARY W. MILLER, AND INGRID LORBACH

(From the La Rabida-University of Chicago Institute, and the Department of Pathology, University of Chicago, Chicago, Illinois 60649)

A prior autogenous anti-idiotypic antibody response markedly suppressed the complementary idiotypic response and vice versa (1). Interestingly, neither response was diminished when mice were immunized to have simultaneous complementary responses. This apparent lack of reciprocal regulation is examined in the following experiments to determine whether a change in idiotype of the complementary antibodies occurs and/or whether the antibodies produced simultaneously are capable of regulating the reciprocal responses.

As in the previous study, the idiotypic and anti-idiotypic responses are those induced in A/He mice against phosphorylcholine (Pc) and against a Pc-binding myeloma protein. Since antibodies raised against these antigens are directed, at least partially, against a combining site structure of the other, the antibodies are referred to as complementary idiotypes or antibodies (1). Complementary idiotypes combine to quench or neutralize the activities of each other; thus, one complementary antibody can be used to inhibit hemolytic plaque formation by cells releasing the other antibody (2). The emergence of clones producing antibody of a different idiotype is revealed by the failure of a standard or "reference" complementary antibody to inhibit plaque formation by cells releasing the different idiotype (3-5). Using this indicator, we find no evidence in the present experiments that simultaneous complementary responses cause an early or rapid shift in idiotypes.

Antibody also inhibits immunization and clonal expansion of normal cells producing the complementary antibody (6). Presumably, antiserum from mice producing antibody of a different idiotype should be less effective in suppressing immunization. Indeed, we report here that sera from mice immunized simultaneously to have circulating levels of both complementary antibodies is less effective in suppressing plaque formation and immunization; however, the ineffectiveness of such sera is apparently due to the formation of complexes. The experimental findings suggest that the formation of circulating complexes is a result rather than the cause of the failure of reciprocal regulation by complementary idiotypes.

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Abbreviations used in this paper: ARA, anti-receptor antibody to H8; FCA, Freund's complete adjuvant; FCS, fetal calf serum; FIA, Freund's incomplete adjuvant; H8, myeloma protein HOPC-8; H8-SRBC, sheep erythrocytes coated with H8; HBSS, Hanks' balanced salt solution; MEM, minimum essential medium; Pc, phosphorylcholine; PcKLH, p-azophenylphosphorylcholine conjugated to keyhole limpet hemocyanin; Pc-SRBC, sheep erythrocytes coated with Pc; PPC, plaque-forming cells; SRBC, sheep erythrocytes; TNP, 2,4,6-trinitrophenylated.
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Materials and Methods

**Mice.** Mice were A/He females 8-12 wk old purchased from The Jackson Laboratory, Bar Harbor, Maine. BALB/c females of the same age purchased from Cumberland View Farms, Clinton, Tenn., provided cells for cultures.

**Antigens and Immunizations.** A formalin-killed vaccine of *Diplococcus pneumoniae*, R36A, has Pc as a major antigenic determinant which mice respond to; the antibody is predominantly IgM. A comparable response to Pc is induced by p-azophenylphosphorylcholine conjugated to keyhole limpet hemocyanin (PcKLH) (7). The same batches of vaccine and PcKLH were used in all the present experiments. Cultures were immunized with 50 µl vaccine containing \( 10^7 \) organisms. Mice were immunized with PcKLH; either 0.2 ml containing 0.7 mg PcKLH/ml saline given intravenously, or with 0.05 ml of a mixture in adjuvants injected in a hind foot pad. The mixture contained 1 part PcKLH 0.7 mg/ml, 1 part methylated bovine serum albumin 1 mg/ml, and 2 parts Freund's adjuvant. Freund's complete adjuvant (FCA) was used for the first immunization; Freund's incomplete adjuvant (FIA) was used for subsequent immunizations.

A single batch of myeloma protein (H8), produced by the plasmacytoma HOPC-8 in BALB/c mice and purified from ascitic fluid (6), was used in all experiments. The H8 protein is of the same idiotype as the TEPC-15 protein (9). We used H8 in the present experiments because we have been more successful in maintaining the HOPC-8 tumor in a stable form which produces abundant ascites containing large amounts of homogeneous protein. For immunizations, one part H8, 1.2 mg/ml, was homogenized with one part methylated bovine serum albumin and two parts Freund's adjuvant. FCA was used for the first immunization and FIA was used for subsequent immunizations.

**Antibody Titers.** Sera were assayed for antibody against Pc and H8 by agglutination of sheep erythrocytes coated with H8 (H8-SRBC) or with C-polysaccharide extracted from R36A (Pc-SRBC) as previously described (1). Sera were assayed in duplicate against H8-SRBC, and SRBC; titers are recorded as the reciprocal, \( \log_2 \), of the mean (to the nearest whole number) of the highest serum dilution which caused a dispersed pattern of sedimentation of target erythrocytes. Titers to SRBC were always low or absent, and no attempt was made to adjust titers because of "background" antibody to SRBC.

**Plaque-Forming Cells.** The preparation of spleen and lymph node cells and assays for numbers of plaque-forming cells (PFC) to Pc-SRBC and H8-SRBC were done as previously described (1). Each suspension of spleen or lymph node cells was assayed in triplicate or quadruplicate for PFC. Controls always included SRBC which were used alone as target antigen. The magnitude of responses was calculated from counts using the target antigen minus counts against SRBC (which rarely exceeded 1,000 PFC per spleen or 100 per lymph node). Additional controls in key experiments included: (a) cells from mice immunized with Pc assayed against 2,4,6-trinitrophenylated (TNP)-SRBC, and (b) cells from mice immunized with TNP-Ficoll assayed against Pc-SRBC (1). Cross-reactions, either stimulating or inhibiting, were not detected and the results for these controls are not reported. Specificity controls for plaque formation to H8-SRBC were not done in these experiments; in unpublished studies, however, we found that cells from mice immunized to the TEPC-15 myeloma protein did not produce plaques against erythrocytes coated with the IgA myeloma protein produced by the MOPC-315 tumor, and cells from mice immunized with the MOPC-315 protein did not produce plaques against erythrocytes coated with the TEPC-15 protein.

**Inhibition of Plaque Formation.** For inhibition of plaque formation, 50 µl of serum diluted in Hanks' balanced salt solution (HBSS) was added to a mixture of 0.3 ml agarose, 50 µl of a suspension of lymphocytes, and 50 µl of target cells just before the mixture was spread and allowed to gel on glass slides. PFC to Pc-SRBC were counted after the slides were incubated at 37°C for 1 h without complement and 1 h with complement. Slides prepared with H8-SRBC were incubated with developing serum (1) 1 h before incubation with complement. Each serum dilution was tested in triplicate or quadruplicate for each cell suspension assayed. Controls included normal serum, and in key experiments the sera were also tested for inhibition of plaque formation to TNP-SRBC by cells from mice immunized with TNP. Again, data for these controls are not reported since the sera did not affect plaque formation by cells releasing anti-TNP antibody.

**Suppression of Immunization in Vitro.** Spleen cells were cultured in vitro using modifications of the Mishell-Dutton procedure (2, 10); 1.5 X 10^6 cells in 1.0 ml of minimum essential medium containing 10% fetal calf serum (MEM-FCS) were cultured in 35-mm Falcon culture dishes.
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(Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). Sera or other materials tested in culture were diluted in MEM-FCS and sterilized by filtration (Millex 0.45-μm disposable filter units). Additional dilutions were then made in MEM-FCS. Usually, 50 μl of each dilution was added to each of three or four cultures 1 h before cultures were immunized with 50 μl of R36A vaccine. 4 days later the cells were harvested, washed twice in 3.0 ml HBSS, and resuspended in 1.0 ml HBSS. Each culture was assayed for PFC in duplicate or triplicate, using 100 μl of cell suspension per slide. The magnitude of responses of normal A/He spleen cells to immunization with Pc is usually ≤ 20% of that obtained with BALB/c cells. For this reason, except as noted in the text, BALB/c spleen cells were used for cultures.

Antiserum to H8. A single pool of "reference" antiserum to the H8 idiotype was raised in 60 A/He mice each injected six times at weekly intervals with 0.2 ml of H8 in adjuvants prepared as described above; mice were bled 10 days after the final immunization. The serum had an agglutinin titer of 13 to H8-SRBC. For convenience, this pool is referred to as anti-receptor antibody (ARA) since the antibody can be considered as directed against a combining site structure for Pc (though it would be just as logical to refer to the anti-Pc antibody as anti receptor antibody since it functions as an antibody directed against the complementary receptor).

Serum Anti-Pc Ig. Normal BALB/c serum from adult mice contains antibody to Pc of the T15 idiotype (11); to isolate this naturally occurring idiotype, serum was precipitated with 45% saturated ammonium sulfate. The precipitate dissolved in distilled water was passed through an Ultragel AcA-34 column (LKB Produkter, Stockholm, Sweden) to obtain an Ig fraction of ~ 150,000 daltons. The amount of H8 idiotype present in the Ig fraction was determined by inhibition of binding of radiolabeled H8 to ARA in a solid-phase radioimmunoassay (12). This Ig fraction was added to cultures to reverse suppression by ARA (Table VIII).

Calculations and Recording of Data. The number of PFC per slide usually varies twofold or less when multiple slides are prepared from a single suspension of cells (lymph node, spleen, or culture). When results are for pools of cells (Tables II, III, VI, VII), the arithmetic mean of counts on three or more replicate slides is reported. The variability of individual responses (measured by counting plaques on replicate slides for each individual) is a direct function of the magnitude of responses. For reasons previously discussed (13), the means of groups of identically treated mice or cultures are calculated on log-transformed data. Thus, the number of PFCs recorded for groups of similarly treated mice or cultures (Tables I, IV, V, VIII, and in the text) is the antilog of the mean for the group; the log of the mean ± the standard error is given in parentheses.

Results

Preliminary Observations. Prior immunization with TEPC-15 myeloma protein markedly suppressed subsequent immunization with Pc and vice versa (1). These observations were repeated to establish that comparable results were obtained using the present reagents. Five mice were immunized three times at weekly intervals with H8 in adjuvants, and five mice were injected with adjuvants only. 1 week after the third injection, these and five normal mice were challenged intravenously with both Pc-KLH and TNP-Ficoll; responses were measured 4 days later. Mice preimmunized with H8 had 1,050 (3.02 ± 0.12) PFC, whereas the control groups had 20,500 (4.31 ± 0.26) and 18,400 (4.25 ± 0.32) PFC to Pc-SRBC per spleen. The three groups had comparable responses to TNP with 45,000 (4.65 ± 0.32), 56,000 (4.74 ± 0.41), and 39,000 (4.59 ± 0.28) PFC/spleen respectively. In a reciprocal experiment, mice were immunized once a week intravenously with Pc-KLH. Beginning 1 wk after one to three immunizations with Pc-KLH, foot pads were injected with H8 in adjuvants; popliteal lymph nodes were assayed for PFC 4 days after two injections. Preimmunization with Pc beginning 1 wk before immunization with H8 very effectively suppressed the response to H8: responses were 1,800 (3.25 ± 0.64) compared with 22,000 (4.34 ± 0.39) PFC to H8-SRBC/node. Suppression was
similar when challenge with H8 was begun after two or three immunizations with Pc. The ARA raised in A/He mice against H8 effectively suppressed immunization to Pc in both A/He and BALB/c mice. For example, 0.2 ml of ARA was given intraperitoneally to each of four normal adult BALB/c mice, and to each of four normal A/He mice 24 h before i.v. immunization with 0.2 ml Pc-KLH. Four control BALB/c and 4 A/He mice received Pc-KLH only. Mean PFC responses per spleen 4 days later were: <1,000 (2.90 ± 0.11) for treated and 78,600 (4.89 ± 0.07) for control BALB/c mice, and <1,000 (2.74 ± 0.20) for treated and 18,500 (4.26 ± 0.24) for control A/He mice. Also, ARA effectively suppressed immunization to Pc in vitro after absorption on BALB/c serum (previously absorbed with Pc-Sepharose to eliminate the Pc idiotype), but not after absorption with serum from BALB/c mice hyperimmunized to Pc, Table I. Taken together, the findings indicate that suppression by ARA is due to antibody directed against a Pc binding site structure.

Whereas prior immunization with either Pc or H8 markedly suppressed immunization to the other antigen, this was not the case when immunizations with the two antigens were begun simultaneously. This was found to be the case whether the conditions of immunization were adjusted to give comparatively low or high responses to either antigen.

Low Anti-Pc Responses Occurring Simultaneously with High Anti-H8 Responses. – The conditions for this set of experiments were purposely selected to give a high anti-H8 and a low anti-Pc response. This was done with the idea that these conditions would provide “maximum selective pressure” favoring emergence of new anti-Pc idiotypes. A low but reproducible response to Pc was obtained when mice were immunized in one third foot pad with the Pc-KLH in adjuvants. 4 days after two to six weekly immunizations, the draining popliteal lymph node contained 400–2,000 PFC to Pc-SRBC; sera from such mice had no detectable agglutinins for Pc-SRBC. Mice immunized identically with H8 in adjuvants three or more times had 10,000–50,000 PFC to H8-SRBC per draining popliteal lymph node, and sera from such mice had agglutinin titers of 7–12 for H8-SRBC. Neither antigen induced a measurable response in the contralateral node, and neither antigen induced a measurable response to the other antigen in either node. These immunization procedures were then used in a series of experiments of the same basic design: one group was immunized in one foot pad with H8 only, a second group was immunized in the opposite foot pad with Pc only, and a third group was immunized with H8 in one foot pad and with Pc in the other foot pad. Immunizations were repeated every 7 days and responses

2 It was previously shown (1) that large amounts of soluble TEPC-15 myeloma protein injected into mice at the time of immunization with Pc did not suppress immunization to Pc. Similarly, large “paralyzing” doses of Pc-containing C-polysaccharide given 1 or 7 days before immunization with TEPC-15 did not suppress immunization to TEPC-15. Thus, it is very unlikely that suppression produced by prior immunization was due to residual free antigen which combined with and neutralized the second antigen. Also, antibody to Pc given passively suppressed immunization with TEPC-15, and antibody against TEPC-15 given passively suppressed immunization to Pc. Together these results support the contention that prior immunization with one antigen suppresses subsequent immunization with the other antigen because of an active immune response to the antigen used first.
Suppression of Immunization to Pc by ARA Is Due to Antibody Directed against the Anti-Pc Idiotype

| Exp. | Added to cultures* | Dilution of ARA | PFC to Pc-SRBC per culture‡ |
|------|--------------------|-----------------|-----------------------------|
| 1    | ARA                | 1:40            | 87 (1.94 ± 0.20)            |
|      | ARA absorbed*     | 1:40            | 51 (1.71 ± 0.25)           |
|      | ARA                | 1:200           | 478 (2.68 ± 0.15)          |
|      | ARA absorbed*     | 1:200           | 776 (2.89 ± 0.10)          |
|      | Medium             | —               | 1,380 (3.14 ± 0.09)        |
| 2    | ARA                | 1:40            | 138 (2.14 ± 0.09)          |
|      | ARA reabsorbed on MOPC-47A§ | 1:40 | 208 (2.32 ± 0.18)          |
|      | ARA reabsorbed on anti Pc serum§ | 1:40 | 1,621 (3.21 ± 0.09)        |
|      | Medium             | —               | 1,862 (3.27 ± 0.05)        |

* ARA diluted 1:40 in buffered saline was absorbed exhaustively on a column of normal BALB/c serum coupled to Sepharose; the BALB/c serum had been previously absorbed with Pc-Sepharose to eliminate the Pc idiotype; 50 µl was added to each culture.
‡ All cultures were immunized with R36A vaccine and assayed 4 days later. PFC to Pc-SRBC per culture were calculated from counts on nine slides prepared in triplicate from three cultures.
§ Aliquots of absorbed serum, exp. 1, were reabsorbed on pooled serum from BALB/c mice hyperimmunized to Pc or on a non-Pc binding IgA myeloma protein, MOPC-47A, coupled to Sepharose.

were measured 4 days after three to six immunizations. The results of a typical experiment using cells and sera pooled from groups of five mice are reported in Tables II–IV. The mean responses to Pc and H8 were equivalent whether mice were immunized with one or both antigens; sera from the groups immunized with H8 had high antibody titers to H8, but sera from groups immunize with Pc had no detectable antibody to Pc (Table II). Plaque formation to Pc-SRBC by cells from mice immunized with Pc only (group 2), or by cells from the appropriate nodes of mice immunized with both Pc and H8 (group 3), was equally inhibited by ARA or by serum from mice immunized with H8 only (group 1), or by serum from mice immunized with both Pc and H8 (group 3, Table III). Also, the pooled sera from mice immunized with H8 only or serum from mice immunized with both antigens produced equivalent suppression of immunization in vitro (Table IV). Results comparable to those reported in Tables II–IV were obtained in two other experiments of the same design, and also in two experiments in which pools of cells and sera were obtained from mice after four or six immunizations.

The experiments did not rule out the possibility that individual mice produced different anti-H8 and anti-Pc idiotypes. If this had been the case, then pooled serum could inhibit plaque formation by cells producing anti-Pc antibody of non-H8 idiotype. Therefore, experiments were designed to test whether lymph node cells from individual mice immunized to both Pc and H8 were susceptible to suppression by their own anti-H8 antibody, as were cells from individual mice immunized with Pc only. Because the responses to Pc were low, it was not possible to prepare more than 10 slides using cells obtained from an immunized lymph node of a single mouse and still have reasonable numbers of PFC per slide. Working with this restriction, sera and cells from the lymph nodes
TABLE II

Complementary Responses in Popliteal Lymph Nodes

| Group* | Immunization | Response to H8‡ | Response to Pc$ |
|--------|-------------|----------------|-----------------|
|        |             | PFC per lymph node | Serum antibody titer | PFC per lymph node | Serum antibody titer |
| 1      | H8          | 16,500          | 9               | –                | 0               |
| 2      | Pc          | –               | 0               | 1,040           | 0               |
| 3      | H8 and Pc   | 14,450          | 8               | 1,440           | 0               |

* Groups of five mice were immunized three times at weekly intervals: group 1 in the right hind foot pad with H8, group 2 in the left hind foot pad with Pc-KLH, and group 3 in the right hind foot pad with H8 and the left hind foot pad with Pc-KLH. Both antigens were in adjuvants.

‡ Mice were sacrificed 4 days after the third immunization. The results are for pools of cells assayed in triplicate or sera assayed in duplicate.

TABLE III

Inhibition of Plaque Formation by Sera from Mice Immunized with H8 or with Both H8 and Pc Using Lymph Node Cells Immunized to Pc

| Sera* | Lymph node cells immunized to Pc from mice in:* |
|-------|-----------------------------------------------|
|       | Group 2 (immunized with Pc) | Group 3 (immunized with Pc and H8) |
| Source | Dilution | PFC per slide | Suppression | PFC per slide | Suppression |
|--------|----------|---------------|-------------|---------------|-------------|
| 1:10   | 10       | 90            | 48          | 67            |
| 1:100  | 17       | 84            | 40          | 73            |
| 1:1,000| 68       | 35            | 88          | 39            |
| 2:10   | 2        | 98            | 0           | 100           |
| 1:100  | 30       | 71            | 64          | 56            |
| 1:1,000| 96       | 8             | 104         | 28            |
| 1:500  | 51       | 49            | 75          | 52            |
| 1:10   | 93       | 11            | 124         | 14            |
| None   | 104      | 0             | 144         | 0             |

* The sera and lymph node cells are the pools from the groups reported in Table II. PFCs per slide are means for counts on three or four slides.

N.S., normal A/He serum.

immunized to Pc were obtained from each of four mice immunized three times with both H8 and Pc, and from each of four mice immunized three times with Pc only. The serum from one mouse immunized with both antigens was tested for inhibition of plaque formation by cells from that mouse and by cells from one mouse immunized with Pc only. Each lymph node cell suspension was assayed against Pc-SRBC in triplicate with each of three sera: (a) the donor serum diluted 1:100, (b) ARA diluted 1:500, and (c) normal serum diluted 1:100. Each donor serum and ARA produced 57–85% inhibition and, in each instance, mean inhibition by a donor serum of ARA was equivalent whether lymph node
TABLE IV
Suppression of Immunization to Pc in Vitro by Sera from Mice Immunized with H8
Alone or with Both H8 and Pc

| Sera from:* | Dilution | Mean PFC per culture | Suppression |
|-------------|----------|----------------------|-------------|
| Group 1 (immunized with H8) | 1:5 | 301 (2.48 ± 0.22) | 63% |
| | 1:25 | 437 (2.64 ± 0.20) | 52% |
| | 1:125 | 794 (2.90 ± 0.16) | 13% |
| Group 3 (immunized with Pc and H8) | 1:5 | 257 (2.41 ± 0.31) | 72% |
| | 1:25 | 347 (2.54 ± 0.05) | 62% |
| | 1:125 | 691 (2.84 ± 0.10) | 24% |
| ARA | 1:500 | 269 (2.43 ± 0.11) | 71% |
| N.S.§ | 1:5 | 1,071 (3.03 ± 0.06) | - |
| Medium | | 912 (2.96 ± 0.04) | 0%

* The sera are pools obtained from the groups, Table I; 50 μl of a dilution of serum was added to each of 4 cultures.
† Each culture was immunized with R36A vaccine after addition of serum or medium. PFC per culture were calculated from counts on nine slides prepared in triplicate from three cultures.
§ N.S., normal A/He serum.

Thus, with simultaneous immunizations the anti-Pc PFC response was not affected even though: (a) plaque formation by the cells releasing anti-Pc antibody was demonstrably inhibitable by anti-idiotypic antibody, and (b) large amounts of such antibody, capable of both inhibiting plaque formation and suppressing immunization in vitro, were present in the serum.

"High" Anti-Pc Responses Occurring Simultaneously with High Anti-H8 Responses. — The above experiments should have been optimal for demonstrating new anti-Pc idiotypes if they emerged within the first weeks of simultaneous responses; it remained possible, however, that high anti-Pc as well as high anti-H8 responses might be more favorable for the appearance of new idiotypes. Therefore, experiments of the same design as described above were repeated, except that mice were immunized to Pc by injecting them intravenously with Pc-KLH in saline. With this route and form of antigen, PFC responses to Pc were higher, and serum contained antibody to Pc. Again, simultaneous immunization with H8 did not diminish the PFC response to Pc, though serum antibody levels to Pc were reduced significantly (Table V). ARA produced equivalent inhibition of plaque formation whether spleen cells were obtained from mice immunized with one or both antigens (Table VI). However, pooled serum from mice immunized with both antigens was relatively ineffective in inhibiting plaque formation to Pc-SRBC using cells from mice immunized to Pc (exp. 1, Table VII).

Results comparable to those reported in Tables V and VI were obtained in three additional experiments; also, in these experiments pooled sera from mice
### TABLE V

*Complementary Responses in Spleens and Popliteal Lymph Nodes*

| Group* | Immunization          | Responses to H8† |                  | Response to Pc† |                  |
|--------|-----------------------|------------------|------------------|------------------|------------------|
|        |                       | PFC per lymph node | Serum antibody titer | PFC per spleen | Serum antibody titer |
| 1      | H8                    | 19,500 (4.29 ± 0.23) | 10 (10-12) |          |                 |
| 2      | Pc                    | -                | 0               | 5,250 (3.72 ± 0.32) | 7 (5-8) |
| 3      | H8 and Pc             | 25,700 (4.38 ± 0.19) | 10 (9-11) | 3,390 (3.54 ± 0.32) | 3 (1-4) |

* Groups of five mice were immunized three times at weekly intervals: group 1 in hind foot pads with H8, group 2 intravenously with Pc-KLH, and group 3 in hind foot pads with H8 and intravenously with Pc-KLH.

† Mice were sacrificed 4 days after the third immunization. Results were calculated from the individual PFC and antibody responses of mice. Differences between groups for PFC responses to Pc and H8, and antibody responses to H8 were not significant; differences between groups for antibody responses to Pc are significant, $P = <0.01$.

### TABLE VI

*Plaque Formation Was Equally Inhibited by ARA Whether Cells Were from Mice Immunized with PC Only or from Mice Immunized with Both Pc and H8*

|                  | Group 2 spleen cells* | | Group 3 spleen cells* | | |
|------------------|-----------------------|------------------|-----------------------|------------------|
|                  | (mice immunized with Pc) | (mice immunized with Pc and H8) | | |
|                  |                       | PFC per slide | Suppression | PFC per slide | Suppression |
| N.S. 1:100§ 1:500 | ARA                   |               | N.S. 1:100 | ARA                   |               |
|                  |                       |               | %          |               | %          |
| 286              | 197                   | 31            | 117        | 59            | 50         |
| 182              | 46                    | 49            | 89         | 85            | 5          |
| 144              | 74                    | 75            | 50         | 14            | 72         |
| 49               | 26                    | 47            | 46         | 19            | 59         |
| 32               | 15                    | 53            | 21         | 9             | 57         |
| Mean = 51       |                       |               |            | Mean = 49     |            |

* Spleen cells were obtained from the individual mice, groups 2 and 3, Table V. The number of PFC per slide is the mean for counts on four slides prepared from an individual mouse.

§ N.S., normal A/He serum.

Immunized with both antigens were relatively ineffective in inhibiting plaque formation to Pc-SRBC (exp. 2–4, Table VII). Sera which were ineffective for inhibiting plaque formation were also ineffective in suppressing immunization to Pc. For example, 50 μl of dilutions of the pooled sera from groups of mice in experiments 1 and 4, Table VII, were added to cultures of normal BALB/c spleen cells 1 h before immunization to Pc. Group 1 sera (immunized to H8 only) from both experiments diluted 1:100 caused >80% suppression, whereas group 3 sera (immunized to both Pc and H8) from both experiments diluted 1:10 or 1:25.
TABLE VII

| Exp. | Mean number of PFC to Pc-SRBC/slide* | Suppression |
|------|-------------------------------------|-------------|
|      | Group 1 serum (immunized to H8)     | Group 3 serum (immunized to Pc and H8) | Group 1 serum* | Group 3 serum* |
| 1    | 72 15 65                             | 79 10       |
| 2    | 85 35 80                             | 58 6        |
| 3    | 24 6 24                              | 75 0        |
| 4    | 180 38 165                           | 79 8        |
| Mean | 73 6                                |             |

* The spleen cells were pools. For exp. 1, the cells and sera were pooled from the individual mice reported in Table IV. Experiments 2, 3, and 4 were identical to exp. 1. In each experiment, pooled sera were tested using pooled group 2 spleen cells obtained in that particular experiment. The number of PFC per slide is the mean for counts on three slides. The difference in suppression produced by group 1 and 3 sera in the four experiments is significant (P < 0.01).

† N.S., normal A/He serum.

caused >80% suppression, and diluted 1:100 caused <10% suppression. (The P value for differences between responses of cultures treated with group 1 and 3 sera diluted 1:100 was <0.01 in both experiments.)

The relative ineffectiveness of sera from mice immunized with both antigens to suppress might be due to some qualitative change in antibody released by cells immunized to H8. This possibility was tested by using antisera to Pc obtained in experiments 3 and 4, Table VII, to inhibit plaque formation by cells immunized to H8; thus, 50 μl of either normal serum or the group 2 serum (i.e. immunized to Pc) diluted 1:500, 1:2,500, or 1:12,500 was added per slide of lymph node cells assayed against H8-SRBC. Each serum dilution was tested in triplicate and the percent inhibition was calculated from the counts obtained with normal serum diluted 1:500. In experiment 3, group 2 serum diluted 1:2,500 inhibited plaque formation of group 1 lymph node cells by 78% and group 3 lymph node cells by 74%. In experiment 4, group 2 sera diluted 1:2,500 inhibited plaque formation of group 1 lymph node cells by 41% and group 3 lymph node cells by 43%. In both experiments, dilutions of 1:500 caused >90% inhibition and dilutions of 1:12,500 caused <10% inhibition. Thus, insofar as it was tested, plaque formation by cells releasing anti-H8 antibodies was equally inhibited by anti-Pc antibody whether cells were obtained from mice immunized with H8 only, or from mice immunized with both H8 and Pc.

In these experiments antibody released by cells in vitro was not detectably different whether cells came from mice immunized with one, the other, or both antigens, though serum from mice immunized with both antigens was relatively ineffective in inhibiting plaque formation and suppressing immunization to Pc. This latter finding could be due to the formation of complexes of the complementary antibodies. The fact that titers of antibody to Pc were significantly lower in
mice immunized to both antigens, Table V, was compatible with this possibility, and the following observations add support to it.

**Complexes in Sera from Mice Immunized with Both Antigens.**—Sera having antibody activity against both Pc and H8 should contain complexes of the two antibodies. The existence of such complexes was indicated in the following way. Putative complexes were formed by mixing ARA diluted 1:20 with an equal volume of antisera from A/He mice immunized three times with R36A vaccine. The diluted ARA had a titer of 8 to H8 and the antiserum to Pc had a titer of 6 to Pc; after incubation overnight at 4°C, the mixture had a titer of 8 to H8 and 5 to Pc. Thus, the mixture retained both antibody activities. Aliquots of the original 2 sera and of the mixture containing putative complexes were each absorbed with an equal volume of packed Pc-SRBC or H8-SRBC for 30 min at 4°C. Absorption of the mixture with H8 reduced the titer to Pc from 5 to 3, and absorption with Pc reduced the titer to H8 from 8 to 4. Titers of the two original sera were reduced only by absorption with the antigen that the antiserum was directed against. Also, the pooled sera from the groups reported in Table V were absorbed in the same way with either Pc or H8, and then titered against the two antigens. The absorption of group 3 serum (mice immunized with both Pc and H8) with either Pc-SRBC or H8-SRBC eliminated the titer to Pc and reduced the titer to H8 from 11 to 8, whereas titers of group 1 and 2 sera were only reduced by absorption with the antigen with which the donor mice were immunized. Thus, the effects of the absorptions on titers were equivalent whether appropriate sera were mixed in vitro to form complexes, or whether sera came from mice immunized with both antigens. These findings suggested that complexes, presumably in equilibrium with free antibody, retain some available combining sites so that absorption with either antigen removed both antibodies.

**Partial Reversal of Suppression by Antibody to Pc.** Apparently, when mice are simultaneously immunized to have high levels of complementary antibodies, complexes form which are ineffective immunosuppressants. If this is the case, then antibody to Pc should prevent suppression by ARA. To test this possibility, purified H8 protein or an Ig fraction of serum containing antibody to Pc was added to cultures just before addition of ARA and R36A vaccine. The Ig fraction rather than whole serum was used because whole serum free of antibody to Pc contains factors which enhance the response of cultures to Pc. PFC responses were measured 4 days later. The highest concentration of H8 tested caused suppression, and this quantity of H8 was ineffective in reversing suppression; quantities of H8 or the Ig fraction, however, which alone neither enhanced nor suppressed significantly, partially prevented suppression by ARA (Table VIII). The serum Ig fraction was tested in a series of experiments at a single concentration of 266 ng of idiotype per culture. Suppression of >90% produced by ARA was reduced to <50% in all experiments. In addition, absorption of the fraction with Pc coupled to Sepharose eliminated the capacity of the fraction to partially reverse suppression by ARA.

**Discussion**

Complementary responses induced simultaneously in adult mice did not cause rapid or gross emergence of clones of different anti-Pc idiotype. This
conclusion at least can be drawn using the same procedure which demonstrated emergence of different idiotypes in neonatally suppressed mice (4, 5), i.e., inhibition of plaque formation by complementary idiotype. Of course, the effectiveness for resolving different idiotypes using anti-idiotypic antibody must be a function of the homogeneity of idiotypes used for inhibition. Though A/He mice have the repertoire to produce various anti-Pc idiotypes, the response of normal adults is predominantly of the H8 (T15) idiotype (14, 15). Less is known about the heterogeneity of anti-idiotypic antibodies raised against myeloma proteins, though purified A/He antibody to H8, prepared from the same pool as used in these studies, is of highly restricted class and spectrotype (16), as is BALB/c antibody raised against MOPC-168 myeloma protein (17). Also, the observation that antisera to each antigen caused equivalent inhibition of plaque formation by cells immunized to produce the complementary antibody suggests that both responses are of similar heterogeneity. It seems reasonable to assume, therefore, that if appreciable changes in idiotypes occurred, we would have
detected such changes by measuring inhibition of plaque formation by cells from mice immunized to have simultaneous complementary responses. This assumption is further supported by demonstrating that sera from individual mice immunized with both antigens (but to have a low response to Pc) suppressed plaque formation by the individual's own lymph node cells immunized to Pc.

It is true that serum from mice immunized to have high simultaneous responses was relatively ineffective in inhibiting plaque formation and suppressing immunization to Pc; this finding might be interpreted as evidence for a change in idiotype of anti-H8 antibody. However, plaque formation by lymph node cells immunized to H8 from these mice was “normally” inhibited by antibody to Pc, and sera from such mice contained immune complexes. In all probability, antibody to Pc neutralized or quenched the activity of antibody to H8 in these sera.

Mice immunized to have low responses to Pc had no measurable agglutinins to Pc; sera from mice immunized in this way to Pc and also to H8 were as effective as sera from mice immunized only with H8 in inhibiting plaque formation and suppressing immunization to Pc. Thus, in mice immunized to have simultaneous low responses to Pc and high responses to H8, the absence of reciprocal regulation cannot be accounted for by the formation of complexes, at least by appreciable levels of circulating complexes. Presumably, the mechanisms preventing reciprocal regulation are the same whether mice had low or high responses to Pc. If this is the case, then the formation of circulating complexes in mice immunized to have high responses to both Pc and H8 is a result rather than the cause for the absence of reciprocal regulation.

Complementary responses induced simultaneously coexist apparently because of the continued absence of reciprocal regulation of the original dominant B-cell clones. Initially, the stimulated clones must proliferate and synthesize antibody in the absence of appreciable complementary idiotype. With progression of responses, released antibody (which can be considered as shed antigen) may shield cells from being targets for complementary idiotype, and in this way prevent regulation. For example, ARA given to mice or added to cultures more than 1 day after immunization with Pc fails to suppress the continued proliferation of cells releasing antibody to Pc (6). However, ARA will suppress if the cells from the mice or cultures are washed thoroughly (D. A. Rowley, unpublished observations), presumably because free anti-Pc antibody is removed. But other mechanisms are probably involved at later stages after immunization. For example, we have found that (FAB)_1, fragments prepared from ARA (which retain full combining activity with the H8 idiotype) do not suppress immunization to Pc in vitro (12). This suggests that cross-linking of surface Fc and Ig receptors may be required for suppression. Responding cells may differentiate and mature to stages which are not as susceptible to regulation because of change in density or distribution of Fc or Ig receptors, or because of loss of interaction between these receptors (18–21). Alternatively, it is possible that complexes of antigen-antibody or antibody-antibody formed on or near the surface membrane of responding cells binds to Fc receptors to prevent regulation by free complementary antibody.

Regulation may be mediated by cells as well as by antibody; thus, it is
possible that the absence of reciprocal regulation may be a result of a change affecting either regulatory cells and/or the susceptibility of responding B cells to regulatory cells. But once a response develops beyond some critical stage beginning about 24 h after immunization, specific suppression mediated by complementary antibody or suppressor cells is apparently much less effective. Presumably, this occurs so that an ongoing or secondary response is not prematurely or inappropriately turned off. Thus, the absence of reciprocal regulation in the present model may be a manifestation of a usual maturation process.

Though simultaneous complementary responses were induced intentionally and quite artifically in these experiments, the potential may exist in nature for other antigens and antibodies to stimulate comparable responses. Antibodies or antigen-antibody complexes can induce anti-idiotypic responses, and the inference is that complexes formed in vivo may account for induction of autogenous anti-idiotypic antibodies (22–31). Also, preliminary studies in our laboratory suggest that some complex antigens may contain complementary determinants, each of which stimulates directly one of the complementary antibody responses (D. A. Rowley, unpublished observations). But regardless of the mechanisms, simultaneous complementary responses may cause untoward effects, e.g., tissue deposition of complexes and activation of complement components. These possibilities are being investigated in our laboratory.

Summary

Complementary antibodies, i.e. antibodies having combining site structures which are at least partially directed against each other, were induced in A/He mice by immunization with phosphorylcholine (Pc) coupled to keyhole limpet hemocyanin or with the Pc-binding IgA myeloma protein, HOPC-8 (H8). Both responses were monitored by enumerating plaque-forming cells and assaying serum antibody levels to Pc and H8. Prior immunization with H8 markedly suppressed subsequent immunization with Pc and vice versa; neither plaque-forming cell response was diminished, however, when mice were immunized simultaneously with Pc and H8.

Experiments were designed to determine if the absence of reciprocal regulation was due to change in idiotypes. This was determined by measuring inhibition of plaque formation using complementary antibody. Plaque formation by cells was equally inhibited by high dilutions of the appropriate complementary antibody whether cells were from mice immunized with one, the other, or both antigens. Thus, the absence of regulation could not be accounted for by emergence of different idiotypes. Interestingly, sera from mice immunized to have high responses to both antigens were relatively ineffective in inhibiting plaque formation or suppressing immunization to Pc. However, such sera contained complexes of the complementary antibodies; apparently antibody to Pc in such sera quenches or neutralizes the activity of anti-H8 antibody. But the formation of complexes, at least measurable levels of circulating complexes, must be a result rather than the cause for the absence of reciprocal regulation, since regulation was also absent when immunization to Pc was manipulated so that responses were too low to result in detectable levels
of circulating antibody to Pc. It is proposed that simultaneous complementary responses may occur in nature to other antigens and antibodies, and that such simultaneous responses may cause pathologic changes.

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