sensitive to complement than adult cells or some other factor must be defined to account for the different effects of ARA on neonatal and adult mice.
24. T. McKearn, M. Neu, F. Fitch, F. Stuart, *Fed. Proc.* **33**, 811 (1974); T. McKearn, F. W. Fitch, F. P. Stuart, *J. Immunol.*, in press; L. Kluskens, H. Köhler, D. Kaplan, S. Smyk, *Fed. Proc.* **33**, 599 (1974); L. Kluskens and H. Köhler, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
25. D. A. Rowley and F. W. Fitch, *J. Exp. Med.* **121**, 683 (1965).
26. We thank S. Smyk, I. Nebl, and W. Hopkins for their skillful assistance. Supported by PHS grants AI-11080, AI-09268, and AI-10242; by Medical Science Training Program trainee awards to D.S.S. and W.M.F.L. under PHS grant 2TO5GMO 1939-06; and by PHS research career development award AI-70559 to H.K.

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Clonal Depletion in Neonatal Tolerance

Abstract. *Specific unresponsiveness can be induced in neonatal and adult BALB/c mice by antibody against antigen-specific receptor (antireceptor antibody). When heterologous antireceptor antibody is used in the indirect fluorescence technique, the number of fluorescent cells in these animals is significantly lower than in normal animals. Fluorescent cells appear after a relatively brief incubation of cells from adult-suppressed animals, whereas no fluorescent cells are detected when cells from neonatally treated animals are incubated briefly. Evidently, treating neonatal mice with antireceptor antibody specifically depletes the antigen-responsive clone. In contrast, antireceptor antibody causes reversible blockade of responsive cells in adult-suppressed animals.*

BALB/c mice can be made specifically unresponsive to phosphorylcholine-containing antigens (PC) by antibody directed against the antigen-specific receptor (ARA) (*1*). As stated in the preceding report (*2*), animals treated with ARA as neonates remain unresponsive for many months, and their cells do not respond in vitro or after adoptive transfer (*2*).

In contrast, mice given ARA as adults begin to respond after 1 week and spleen cells removed at any time after ARA injection produce antibodies against PC in vitro. These findings may be explained by assuming that different mechanisms operate in newborns and adults: (i) depletion of the responsive clone in neonatally induced unresponsiveness and (ii) blockade of the receptors for PC on the responsive clone in adult-suppressed animals. If this hypothesis is correct, spleens from adult-treated animals should contain normal numbers of receptor-bearing cells, whereas far fewer such cells should be present in spleens of neonatally treated animals.

To examine these possibilities we have taken advantage of the finding that the response of BALB/c mice to PC is monoclonal (*3*, *4*). We enumerated the cells bearing receptors for PC by indirect immunofluorescence using a heterologous ARA. Neonatally suppressed animals are devoid of these cells, whereas in adult-suppressed animals they are demonstrable after brief incubation.

Suppression of neonatal and adult mice has been described (*2*). Heterologous ARA was prepared in rabbits by immunization with purified BALB/c myeloma TEPC-15, which binds phosphorylcholine. This serum was absorbed on Sepharose to which normal BALB/c serum and purified BALB/c myeloma protein MOPC-21 (immunoglobulin A, kappa) had been coupled. The specificity of the heterologous ARA was established as follows. The binding of ^{125}I-labeled purified TEPC-15, measured by indirect radioimmune precipitation (*5*), could be inhibited only by unlabeled TEPC-15 protein. Normal BALB/c serum and myeloma proteins McPC-167, MOPC-603, and MOPC-21 did not inhibit even at a 20-fold higher concentration. Thus, solid absorption rendered the rabbit antiserum to TEPC-15 (anti-TEPC-15) specific for the TEPC-15 protein. This absorbed heterologous ARA had biological activity similar to that of homologous ARA. For example, it completely inhibited the response to PC when added to cultures of spleen cells in a final dilution of 1 : 50, but it did not inhibit the response to sheep red blood cells (SRBC). Also, this ARA produced suppression of responses to PC in adult and neonatal mice equivalent to that produced by homologous ARA.

Suspensions of spleen cells from normal or suppressed mice were purified on a Ficoll-Hypaque gradient (*6*). The cells were washed three times and processed for indirect fluorescence staining either immediately or after incubation in culture medium (*7*). The fluorescence staining was done at 4°C in the presence of azide (1 mg/ml). Cells were incubated with the ARA diluted 1 : 10, washed, and further incubated with fluorescein-conjugated goat antiserum to rabbit immunoglobulin (goat antirabbit serum) (Meloy) which had been absorbed with normal BALB/c spleen cells. The cell concentration was adjusted so that the 3 by 3 mm^2 scored portion of a standard hemocytometer chamber contained at least 1.5×10^3 nucleated cells. Cells were first counted under dark-field illumination, and then fluorescent cells in the same chamber were counted under Ploem illumination (*8*, *9*). A patchy distribution of the label was seen on most of the fluorescent cells.

Spleen cell suspensions from normal adult animals contained about 30 to 45 fluorescent cells per 10^4 cells (Table 1). Very few fluorescent cells were found in spleens freshly removed from mice suppressed as newborns or as adults. If the absence of fluorescent cells in spleens from suppressed animals was caused by covering of receptors by ARA, then these receptor molecules and the administered homologous ARA might be shed, and new receptors synthesized during incubation. Therefore,

Table 1. Number of cells of normal and suppressed mice stained by indirect fluorescence. Adult animals received homologous ARA 1 week before fluorescence examination; neonatal animals received this serum 2 months before examination. Single spleen cell suspensions were incubated with solid-absorbed rabbit anti-TEPC-15 or normal rabbit serum in 5 percent bovine serum albumin in phosphate-buffered saline with 1 percent azide at 4°C for 30 minutes. After three washings the cells were further incubated with fluorescein-conjugated goat antirabbit serum for 30 minutes and then washed again. The number of cells in a hemocytometer chamber was first determined under dark-field illumination; then the number of fluorescent cells in the same chamber was counted under Ploem illumination. The range in number of fluorescent cells recorded is derived from counts on three or four different preparations of cells. For each preparation, cells were counted in five to ten hemocytometer chambers with each chamber containing at least 1.5×10^3 nucleated cells.

Source of cells	Serum	Fluorescent cells per 10^4 spleen cells
Normal BALB/c	Normal rabbit	< 1
Normal BALB/c	Rabbit ARA	30–45
BALB/c suppressed as adult	Rabbit ARA	< 1
BALB/c suppressed as neonate	Rabbit ARA	< 1

Table 2. Effect of incubation on the number of fluorescent cells. Dissociated spleen cells were incubated in Mishell-Dutton culture medium at 37°C for up to 4 hours. After each hour the medium was changed and the cells were incubated in fresh medium. Other procedures were as described in legend to Table 1.

Source of cells	Incubation time	Fluorescent cells per 10^5 spleen cells
BALB/c suppressed as adult	5 minutes	<1
BALB/c suppressed as adult	4 hours	25–35
BALB/c suppressed as neonate	5 minutes	<1
BALB/c suppressed as neonate	4 hours	<1

cells from suppressed animals were incubated a second time for 4 hours in medium that was changed every hour. The cells were then stained by the indirect fluorescence technique and examined. In cell suspensions from adult-suppressed animals, fluorescent cells could then be detected. Very few such cells were found in the suspension obtained from neonatally suppressed mice (Table 2).

When adult mice were suppressed with heterologous rabbit ARA 24 hours before examination for surface fluorescence, the presence of the administered ARA on receptor cells could easily be detected by staining with fluorescein-conjugated goat antirabbit serum. In this experiment the cell suspensions were screened without the Ficoll-Hypaque centrifugation step, and the cells were only washed once. Again, incubating and changing medium decreased the number of fluorescent cells drastically. When these cells, after the first incubation in medium, were incubated with ARA and fluorescein-conjugated goat antirabbit serum, fluorescent cells could be detected again.

Typically, the number of cells binding a particular hapten such as the dinitrophenyl group in nonimmunized animals is approximately 30 to 50 per 10^5 cells (*10*). We could demonstrate a marked difference between the number of fluorescent cells from suppressed and normal animals. To rule out the possibility that the heterologous ARA (anti-TEPC-15) reacted with cells other than those of the TEPC-15 clone, we added purified TEPC-15 myeloma protein or BALB/c antibody against PC (*11*) during incubation with anti-TEPC-15. Only a few faintly fluorescent cells were detected after this treatment. These cells represent the lowest background we could attain, and they limit our ability to determine whether receptor-bearing cells are completely absent. However, we conclude that solid absorption of the heterologous anti-TEPC-15 rendered this serum sufficiently specific to allow detection of differences in the number of PC-responsive cells in groups of animals treated differently. By these criteria, it seems that the size of the receptor-bearing clone in normal BALB/c mice is at least one order of magnitude larger than has been found for other antigens (*12, 13*). These mice are evidently under weak but constant stimulation by PC in the normal gut flora (*14*). This notion is confirmed by the absence of fluorescent cells in germ-free animals (*15*).

Our ability to detect a specific antigen-responsive clone by immunofluorescence allowed us to study the effect of experimental manipulations on that clone of immunocompetent cells. Animals treated with ARA as newborns remained unresponsive to PC for more than 8 months (*2*). We could detect very few fluorescent cells in spleens of such animals. Evidently, the clone of cells responsive to PC had been depleted.

Suppression of this clone in mature animals yielded only short-term unresponsiveness. If cells from these animals were examined for receptor fluorescence immediately after the spleen was removed, few cells were stained. However, if we allowed time for the cells to shed and regenerate their receptors (*16*), we observed a normal number of fluorescent cells. The unresponsiveness of adult-treated mice probably results from reversible blockade of receptors by ARA; the observation that cells from adult-suppressed animals respond normally to PC in vitro supports this conclusion (*2*).

Persistence of passively given ARA can account for the duration of suppression in adults. The half-life of passively administered isologous immunoglobulin in adult mice is approximately 3 to 5 days (*17*). By 1 or 2 weeks after treatment of BALB/c mice with homologous ARA, some treated mice responded almost normally to PC, but by 4 weeks almost all mice responded normally to PC. Therefore, the time of reappearance of the response to PC in adult-suppressed animals is compatible with the half-life of administered mouse immunoglobulin.

Neonatal unresponsiveness lasting more than 8 months cannot, however, be explained by persisting ARA. We have found that the half-life of ^{125}I-labeled immunoglobulin G in newborn mice is about 7 days. Taken together, this fact and the data presented here and in the accompanying report (*2*) preclude reversible receptor blockade and favor clonal depletion as a possible mechanism of neonatally induced suppression.

Neonatally induced tolerance and naturally occurring discrimination of "self" and "not self" have been explained by a process of selective depletion of responsive clones (*18*). We believe that unresponsiveness induced in neonates by ARA (*2*) represents an experimental model for selective clonal depletion.

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References and Notes

1. H. Cosenza and H. Köhler, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2701 (1972).
2. D. S. Strayer, H. Cosenza, W. M. F. Lee, D. A. Rowley, H. Köhler, *Science* **186**, 640 (1974).
3. H. Cosenza and H. Köhler, *ibid.* **176**, 1027 (1972).
4. W. Lee, H. Cosenza, H. Köhler, *Nature (Lond.)* **247**, 55 (1974).
5. B. W. Brient, J. Haimovich, H. Nisonoff, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 3136 (1971).
6. A. Böyum, *Scand. J. Clin. Lab. Invest.* **21** (Suppl. 97), 77 (1968).
7. R. I. Mishell and R. W. Dutton, *Science* **153**, 1009 (1966).
8. J. S. Ploem, *Ann. N.Y. Acad. Sci.* **177**, 414 (1971)
9. We checked the feasibility of this approach by comparing the number of tumor cells producing myeloma protein (TEPC-15) with the number of cells bearing TEPC-15 receptors on their surfaces. By indirect immunofluorescence and indirect plaque formation, the numbers of these cells in the same suspension were found to be equal [P. Beatty, D. Kaplan, H. Köhler, in preparation].
10. J. M. Davie and W. E. Paul, *J. Exp. Med.* **134**, 495 (1971).
11. J. C. Scornik, L. Kluskens, H. Köhler, *Fed. Proc.* **32**, 989 (1973).
12. G. L. Ada and G. L. Byrt, *Immunology* **17**, 503 (1969).
13. D. Naor and D. Sulitzeanu, *Nature (Lond.)* **214**, 687 (1967).
14. M. Potter, *Physiol. Rev.* **52**, 631 (1972).
15. R. Braylan and J. C. Scornik examined frozen spleen sections from normal and germfree mice, using heterologous ARA and fluorescein-conjugated goat antirabbit serum. They found no fluorescent cells in sections taken from spleens of germfree mice, while sections from normal mice contained a small number of fluorescent cells.
16. E. S. Vitetta and J. W. Uhr, *J. Exp. Med.* **136**, 676 (1972).
17. H. L. Spiegelberg and B. O. Weigle, *ibid.* **121**, 323 (1965).
18. R. E. Billingham, L. Brent, P. B. Medawar, *Nature (Lond.)* **172**, 603 (1953).
19. Supported by PHS grant AI 11080, by NIH research career development award AI 70559 to H.K., and by Medical Science Training Program trainee award to D.S. under PHS grant 2TO5GMO 1939-06.

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