more than a decade in any one region, would be largely invisible to paleontologists. A test of extinction by overkill is in the radiocarbon chronology. The apparently synchronous loss of the Shasta ground sloth with the arrival of big game hunters in Arizona and the slightly younger age of ground sloth remains in South America are in accord with the model (*7*).

AUSTIN LONG
Laboratory of Isotope Geochemistry, Department of Geosciences, University of Arizona, Tucson 85721
PAUL S. MARTIN
Department of Geosciences, University of Arizona

References and Notes

1. The mouth of Rampart Cave can be seen from the Colorado River just inside the Grand Wash Cliffs at the extreme lower end of the Grand Canyon, Mohave County, Arizona. It opens near the base of the Muav limestone about 200 m above river level. The scientific significance of Rampart Cave was recognized in 1936. Surface collections and two test pits yielded abundant bones of *Nothrotheriops*, mainly of young animals [R. W. Wilson, *Carnegie Inst. Washington Publ. No. 530* (1942), p. 171]. There was no evidence of prehistoric human occupation [G. C. Baldwin, *Masterkey* **20**, 94 (1946)]. Our map (Fig. 1) is based on study of the well-preserved face cut in the sloth dung in 1942 by Remington Kellogg of the U.S. National Museum.
2. J. Emperaire and E. Laming, *J. Soc. Am.* **42**, 173 (1954); H. A. Lagiglia, *ibid.* **57**, 161 (1968).
3. P. S. Martin, B. E. Sabels, D. Shutler, Jr., *Am. J. Sci.* **259**, 102 (1961).
4. Occupation was probably confined to the late winter or spring, as suggested by high counts of *Sphaeralcea* and *Ephedra* pollen in the dung (*3*).
5. A younger date on sloth hide from Aden Crater (Y-1163A) is from the same animal dated by Y-1163B. It is considered possibly contaminated by organic preservatives [E. L. Simons and H. L. Alexander, *Am. Antiq.* **29**, 390 (1964)]. Younger dates on sloth dung from Gypsum Cave (C-221, 10,455 ± 340; C-222, 8,527 ± 260) were based on the now superseded solid carbon technique of 25 years ago. Unless they can be replicated, we believe they do not constitute adequate evidence of the postglacial survival of *Nothrotheriops*. The youngest date on sloth dung from Gypsum Cave that we would accept is that of A-1202. The alleged association of sloths and prehistoric people at Gypsum Cave [M. R. Harrington, *Southwest Museum Paper No. 8* (1933)] may be questioned on the basis of 2400 and 2900 year dates on wooden artifacts found beneath sloth dung [R. F. Heizer and R. Berger, *Contr. Univ. Calif. Archaeolog. Res. Facility No. 7* (1970), p. 13]. Wood rat intrusion appears likely.
6. C. V. Haynes, in *Pleistocene and Recent Environments of the Central Great Plains*, O. Dort, Jr., and J. K. Jones, Jr., Eds. (Univ. Press of Kansas, Lawrence, 1970), p. 77.
7. P. S. Martin, *Science* **179**, 969 (1973).
8. Supported by NSF grant GA-16600 to A.L. and NSF grant GB-27406 to P.S.M. and the State of Arizona. R. Brumbaugh, A. Gottesfeld, J. E. King, D. LaRocca, E. Robbins, B. Robbins, and T. Van Devender aided at Rampart Cave; H. Lagiglia, A. Russell, and J. Russell helped collect samples in South America; S. R. Woodmansee identified and quantified the plant fragments in the dung from the sloth caves. D. Evans and other personnel of the U.S. Park Service, Boulder City, Nevada, authorized our research effort, provided access to photographs, collections, and records in their care, and authorized our visits to the caves. This is contribution 68, Department of Geosciences, University of Arizona.

20 February 1974

Neonatal Tolerance Induced by Antibody against Antigen-Specific Receptor

Abstract. *Specific immunologic unresponsiveness is induced by injecting adult or neonatal mice with antibody against antigen-specific receptor (antireceptor antibody). Suppression in mice treated as adults lasts several weeks, and cells from these suppressed mice respond normally in culture. In contrast, unresponsiveness induced in neonatal mice is long-lasting; cells from these mice do not respond in culture and do not affect the response of normal cells. Evidently, antireceptor antibody reversibly blocks antigen receptors in adult animals, but induces unresponsiveness in neonatal mice by depleting the clone of receptor-bearing cells.*

Classically, immunological tolerance is produced by giving antigen to neonatal animals. For antigens that persist, tolerance is long-lasting. Cells from tolerant animals are specifically unresponsive to these antigens when immunized in vitro or after transfer to irradiated syngeneic recipients (*1*). Furthermore, cells from tolerant animals usually do not affect the response of normal cells to the antigen in question (*2*). One hypothesis suggested by these findings, taken together, is that the clones of cells responsive to the antigen producing tolerance have been depleted (*3*).

Adults may be made specifically unresponsive in several ways (*4*), one of which is to give antibody directed against the cell membrane receptor for an antigenic determinant.

The receptor for an antigen and the antibody that the cell produces to that antigen have identical antigen-combining regions. These antigen-binding sites are themselves potentially antigenic; antibody directed against them may be termed antireceptor antibody (ARA) (*5, 6*).

We report here that suppression in adults by ARA is not due to depletion of the receptor-bearing clone. Rather, ARA blocks the interaction between receptors and antigen, and this blockade probably lasts about as long as the passively administered antibody persists. On the other hand, ARA given to neonates produces long-term specific unresponsiveness, and cells from such animals remain unresponsive in vitro and in irradiated hosts. These cells do not suppress the response of normal cells in vitro or in vivo. These results are most readily explained by assuming that ARA depletes the clone of receptor-bearing cells in the neonate. Furthermore, we suggest that this mechanism may be involved in the induction of classic neonatal tolerance produced by antigen.

In our model, ARA is directed against the receptor for the hapten phosphorylcholine. BALB/c mice respond to phosphorylcholine with an immunoglobulin M (IgM) antibody of restricted heterogeneity. The antigen-combining region of this antibody is very similar or identical to the combining region of the phosphorylcholine-binding immunoglobulin A (IgA) protein produced by the BALB/c myeloma TEPC-15 (*7, 8*). The antigen-combining site of this myeloma protein itself serves as an antigen, and elicits antibody to TEPC-15 when injected into A/He mice. This antibody to TEPC-15 (i) neutralizes the specific antibody activity of antibody to phosphorylcholine and of TEPC-15 myeloma protein; (ii) specifically suppresses the response of BALB/c mice and spleen cells to phosphorylcholine; and, therefore, (iii) may be characterized as an ARA (*5, 7*).

The antigens used to immunize against phosphorylcholine were the heat-killed vaccine of R36A strain pneumococcus, or phosphorylcholine diazonium coupled to the protein carriers: keyhole limpit hemocyanin, *Salmonella typhi* flagella, or bacteriophage fd coat protein; all of these antigens induce high responses of antibody of the same idiotype to phosphorylcholine and are referred to as PC (*9*). The different PC's were used to ensure that suppression of response to PC did not depend on the form in which the hapten was presented (*8*). Other antigens used as controls were the trinitrophenyl hapten coupled to a carrier (TNP), sheep erythrocytes (SRBC), and horse erythrocytes (HRBC). Responses to antigens were measured by enumerating cells producing specific antibody by using the plaque-forming cell (PFC) technique of Jerne and Nordin (*9*) as modified for use with glass microscope slides (*10*). Responses to PC were measured by using SRBC coupled to *p*-phenylphosphorylcholine (*11*) or coated with the C-polysaccharide extract obtained from R36A vaccine (*12*). Responses to

TNP were measured by using SRBC coupled to trinitrophenol, and responses to SRBC were determined by using SRBC.

Newborn BALB/c mice received one or two injections of 0.1 ml of ARA intraperitoneally within 1 week of birth (*13*). Adult BALB/c mice received one intraperitoneal injection of 0.3 or 0.4 ml of the same serum. Control animals received either no treatment or normal A/He mouse serum (NMS). Cultures of dispersed spleen cells, 10^7 to 2×10^7 cells in 1.0 ml of medium per culture, were immunized with R36A vaccine (*7*); separate cultures were immunized with TNP or SRBC. The assays for PFC were the same for cultured spleen cells as for spleen cells from intact mice. The kinetics and magnitude of the responses obtained in vitro were comparable to those obtained in vivo (*5*).

In a series of experiments, adult mice ranging in age from 2 to 8 months which had been given ARA as neonates and control mice of similar ages were immunized with PC. Some mice in both groups were immunized also with HRBC or TNP or both. Almost all of the mice in all age groups which had been treated with ARA had markedly reduced responses to PC, but had normal responses to TNP and HRBC (Table 1). Apparently, responsiveness of the neonatally suppressed mice increases slowly with age; for example, the mean response of neonatally suppressed mice immunized when 2 months old was 236 PFC per spleen, whereas the mean response of mice immunized when 8 months old was 3910 PFC per spleen. The responses of control mice 2 to 8 months old when immunized with PC usually varied from 50,000 to 150,000 PFC per spleen.

These findings contrast sharply with those obtained when ARA was given to 2-month-old adult mice. Responses to PC were markedly suppressed when the mice were challenged within a week after receiving ARA, but responses returned to nearly normal levels within a month (Table 2) (*14*).

In a series of in vitro experiments, spleen cells from adult mice treated as neonates with ARA responded poorly to PC, although the cells were fully responsive to control antigens. In contrast, spleen cells taken from adult mice given ARA 1 to 4 days previously (that is, when similar mice had markedly suppressed responses in vivo) responded normally in vitro to both PC and control antigens (Table 3). Presumably, ARA given to adults causes suppression by masking receptors to prevent interaction with antigen. Spleen cells are always washed before being cultured; washing spleen cells obtained from adult mice treated with ARA probably removed the ARA, so that the cells responded normally in vitro. In support of this suggestion, Cosenza (*15*) and Köhler (*16*) found that normal spleen cells incubated with ARA in vitro and then washed before antigen was added responded normally, whereas cells that were not washed did not respond.

The long duration of suppression to PC in neonatally treated mice and the specific unresponsiveness of cells from such mice in culture contrast with the short-term suppression in mice given ARA as adults and the responsiveness of cells from these adult treated mice in vitro. The effects observed in neonatally suppressed mice thus cannot be explained by assuming that the passively administered ARA itself continues to cause suppression. We therefore tested the possibility that suppression might be actively mediated by cells or humoral factors, as has been reported in a different system (*17*). In one series of experiments, spleen cells from neonatally suppressed and normal mice were mixed in vitro in a broad range of ratios while the cell density was kept constant or was varied by up to a factor of 2. The cells were immunized in vitro with PC either immediately or after incubation for 1 day; responses were measured 3 and 4 days after immunization. In no instance was there any evidence that cells from suppressed mice decreased the response of normal cells. For example, cultures of 10^7 cells from suppressed mice had no detectable response, whereas cultures of 10^7 normal cells had a mean of 960 PFC per culture; cultures of a mixture of 10^7 sup-

Table 1. The response in vivo of mice treated as neonates with ARA. Sixty neonatal BALB/c mice were injected with antibody directed against the receptor for phosphorylcholine. They were challenged as follows: 18 mice at 2 months of age, 17 at 3 months, 10 at 4 months, 4 at 5 months, 7 at 6 months, and 4 at 8 months. The 37 control mice received either no injection or 0.1 ml of NMS at birth. Of the controls, 14 were tested at 2 months of age, 12 at 3 months, 4 at 4 months, 2 at 5 months, 4 at 6 months, and 2 at 8 months. At the time of challenge, all mice were injected intravenously with PC; 20 of the neonatally suppressed group and 15 controls were also injected with TNP, and 20 neonatally suppressed and 21 control mice were injected with 2×10^8 HRBC. All responses were measured 4 days after immunization. The responses of mice receiving additional antigen were not significantly different from those of mice receiving only PC. The number of PFC per spleen is the antilog of the mean for individual mice; the log of the mean and the log of the standard error of the mean are given in parentheses. All calculations were performed on logarithmically transformed data.

Mice	Response (PFC per spleen)		
	PC	TNP	HRBC
ARA given neonatally	1,880* (3.275 ± 0.174)	17,000 (4.231 ± 0.047)	190,000† (5.278 ± 0.056)
Control	85,100 (4.930 ± 0.058)	20,800 (4.319 ± 0.087)	284,000 (5.453 ± 0.040)

* $P < .001$. † $P < .02$.

Table 2. The response in vivo of mice treated as adults with ARA. All mice were 6 to 8 weeks old when injected with ARA or NMS, and less than 12 weeks old when immunized. Data presentation and calculations were described for Table 1. Seven additional mice injected with ARA 1 week before immunization with SRBC had a mean of 185,000 (5.275 ± 0.063) PFC per spleen when tested with SRBC; ten additional mice injected with NMS 1 week before immunization with SRBC had a mean of 219,800 (5.342 ± 0.113) PFC per spleen when tested with SRBC. The difference between these two groups is not significant. The specificity of suppression of adult mice by ARA has been reported (*5*); *N*, number of animals.

Treatment	*N*	PC response (PFC per spleen)
ARA 0 to 1 week before antigen	41	1,309* (3.117 ± 0.099)
ARA 2 to 3 weeks before antigen	10	16,320 (4.213 ± 0.274)
ARA 4 weeks before antigen	7	59,730 (4.776 ± 0.220)
NMS 0 to 1 week before antigen	18	131,500 (5.119 ± 0.370)

* $P < .001$ for comparison with mice given ARA 4 weeks before antigen or mice given NMS.

Table 3. Response in vitro of spleen cells from mice given ARA either as neonates or as adults. All spleen cells were obtained from mice 2 to 4 months old. Control cells were from mice either untreated or injected with NMS before cells were cultured. Adult-suppressed animals received 0.3 to 0.4 ml of ARA 1 to 4 days before spleens were removed. Cells from each mouse were cultured separately in triplicate and immunized with PC. Separate additional cultures prepared from each mouse were immunized with TNP or with SRBC. Data presentation and calculations were described for Table 1. Differences in responses to TNP or SRBC are not significant; *N*, number of animals.

Cells	*N*	Response (PFC per culture)		
		PC	TNP	SRBC
Neonatally suppressed	10	89* (1.95 ± 0.16)	389 (2.59 ± 0.19)	2070 (3.32 ± 0.06)
Adult-suppressed	16	646 (2.81 ± 0.11)	1590 (3.20 ± 0.11)	3820 (3.58 ± 0.09)
Control	11	813 (2.91 ± 0.08)	776 (2.89 ± 0.13)	3040 (3.48 ± 0.10)

* $P < .001$ for comparison with adult-suppressed or control cells.

pressed cells and 10^7 normal cells had a mean of 1120 PFC per culture. When suppressed cells or normal cells were cultured alone, they had equivalent high responses to SRBC.

In another series of experiments, spleen cells from mice suppressed as neonates were mixed with equal numbers of normal spleen cells and injected into syngeneic irradiated (600 r) recipients. Recipients were immunized 2 days later and responses were measured 4 days after immunization. Again, in no instance was there any evidence that cells from suppressed mice decreased the response of normal cells. For example, in one experiment recipients were immunized 2 days after they had received 2×10^7 cells from suppressed mice, 2×10^7 normal cells, or 2×10^7 cells of each kind. The mean responses measured 4 days later were 112 PFC per spleen for four recipients of cells from suppressed mice, 5,664 PFC per spleen for four recipients receiving normal spleen cells, and 11,321 PFC per spleen for eight mice receiving both suppressed and normal cells.

The combined results of these experiments are consistent with the assumption that ARA given to neonatal mice depleted the clone of cells reactive with PC and that the clone was reestablished only slowly with age. In contrast, ARA given to adults suppressed by interfering with the interaction between PC and receptors, and suppression lasted about as long as the administered ARA persisted. Data directly supporting this suggestion are presented in the accompanying report (*18*).

The receptor-bearing clone might be depleted by antibody-dependent cell-mediated cytotoxicity (*19*). Cells from normal neonatal mice are more active than those from adults as effector cells (*20*); our ARA causes cytotoxicity of chicken erythrocytes coated with TEPC-15 protein in the presence of neonatal lymphocytes (*21*). "Normal" mouse immunoglobulin G (IgG)—that is, IgG not directed against the specific target—will inhibit antibody-dependent cell-mediated cytotoxicity, presumably by competing for effector cell receptors for the Fc portion of the γ chain of IgG (*22*). The ratio of administered ARA to "normal" IgG is much higher in neonatal mice than in adults. Thus, ARA may deplete the receptor-bearing clone by antibody-dependent cell-mediated cytotoxicity in the neonate, which has very little IgG, whereas it may be much less effective in doing so in the adult because the latter has higher levels of normal IgG. Preliminary experiments support this possibility (*23*).

Antibody-dependent cell-mediated cytotoxicity may be the cause of clonal depletion in antigen-induced neonatal tolerance. An individual may respond to repeated injections of an antigen by producing specific antiantibody, that is, ARA, as well as antibody to the antigen (*24*). Neonatal animals produce small amounts of antibody to tolerance-producing doses of at least some antigens (*25*). This antibody, probably complexed with the antigen producing tolerance, may stimulate production of an ARA that depletes the clone of cells reactive with the antigen. Thus, we suggest that an ARA may be involved in the induction of neonatal tolerance produced by antigen.

DAVID S. STRAYER
HUMBERTO COSENZA
WILLIAM M. F. LEE
DONALD A. ROWLEY
HEINZ KÖHLER

La Rabida Institute and *Departments of Pathology* and *Biochemistry, University of Chicago, Chicago, Illinois 60649*

References and Notes

1. Cells from adult animals made tolerant to an antigen neonatally are unresponsive on adoptive transfer [H. Friedman, *J. Immunol.* **94**, 352 (1965); F. M. Dietrich and W. O. Weigle, *ibid.* **92**, 167 (1964); B. Argyris, *ibid.* **96**, 273 (1966)] and in vitro [R. W. Dutton, *ibid.* **93**, 814 (1964); H. Friedman, *ibid.* **96**, 289 (1966); D. B. Wilson, W. K. Silvers, P. C. Nowell, *J. Exp. Med.* **126**, 655 (1967)].
2. L. Brent, C. Brooks, N. Lubling, A. V. Thomas, *Transplantation* **14**, 382 (1972); W. O. Weigle and F. J. Dixon, *J. Immunol.* **82**, 516 (1959); M. W. Cohen and G. J. Thorbecke, *Proc. Soc. Exp. Biol. Med.* **112**, 10 (1963).
3. There is considerable debate about the mechanism of induction and maintenance of tolerance in neonates [L. Brent, in *Immunological Tolerance to Tissue Antigens*, N. W. Nisbet and M. W. Elves, Eds. (Orthopaedic Hospital, Oswestry, England, 1971); S. Leskowitz, *Annu. Rev. Microbiol.* **21**, 157 (1967); D. W. Dresser and N. A. Mitchison, *Adv. Immunol.* **8**, 129 (1968); W. O. Weigle, *ibid.* **16**, 61 (1973)].
4. D. A. Rowley, F. W. Fitch, F. P. Stuart, H. Köhler, H. Cosenza, *Science* **181**, 1133 (1973).
5. H. Cosenza and H. Köhler, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2701 (1972).
6. D. A. Hart, A. L. Wang, L. L. Pawlak, A. Nisonoff, *J. Exp. Med.* **135**, 1293 (1972); H. Ramsier and J. Lindenmann, *ibid.* **134**, 1083 (1971).
7. H. Cosenza and H. Köhler, *Science* **176**, 1027 (1972).
8. W. M. F. Lee, H. Cosenza, H. Köhler, *Nature (Lond.)* **247**, 55 (1974).
9. N. K. Jerne and A. A. Nordin, *Science* **140**, 405 (1963).
10. P. H. Plotz, N. Talal, R. Asofsky, *J. Immunol.* **100**, 744 (1968).
11. Synthesis of phosphorylcholine diazonium was performed according to the method of B. Cheseboro and H. Metzger [*Biochemistry* **11**, 766 (1972)]. We refer to the hapten phosphorylcholine as "phosphorylcholine," while the various hapten-carrier conjugates are all referred to as "PC." The hemocyanin used was from the keyhole limpit *Megathura crenulata*, and was purchased from Calbiochem, La Jolla, California. We are indebted to Dr. Braunitzer of the Max Planck Institute, Munich, for providing us with the bacteriophage fd coat protein.
12. H. H. Fudenberg, G. Drews, A. Nisonoff, *J. Exp. Med.* **119**, 151 (1964).
13. Neonatal mice were obtained by crossing BALB/c females with $(BALB/c \times A/He)F_1$ or BALB/c males. Untreated adults from both crosses produce equivalent responses to PC. Both sets of animals were used for in vivo experiments, but all experiments involving mixing cells from different animals used only BALB/c × BALB/c mice. For convenience, the notation BALB/c will be used to designate both BALB/c and $BALB/c \times (BALB/c \times A/He)F_1$ mice.
14. In another system, multiple injections of heterologous anti-idiotypic serum caused suppression in some mice which varied from weeks to months depending on several variables [L. L. Pawlak, D. A. Hart, A. Nisonoff, *J. Exp. Med.* **137**, 1442 (1973)].
15. H. Cosenza, *Fed. Proc.* **31**, 752 (1972).
16. H. Köhler, *ibid.*, p. 751.
17. L. A. Herzenberg, E. L. Chan, M. M. Ravitch, R. J. Riblet, L. A. Herzenberg, *J. Exp. Med.* **137**, 1311 (1973).
18. H. Köhler, D. Kaplan, D. Strayer, *Science* **186**, 643 (1974).
19. P. Perimann and G. Holm, *Adv. Immunol.* **11**, 117 (1969).
20. P. Ralph, I. I. Nakoinz, M. Cohn, *Nat. New Biol.* **245**, 158 (1973).
21. D. Strayer, in preparation.
22. J. C. Scornik, H. Cosenza, D. A. Rowley, H. Köhler, *J. Immunol.*, in press.
23. We have found that normal mouse serum given to neonates injected simultaneously with ARA blocks the induction of suppression, possibly because of the free IgG present in NMS. Purified IgG has the same effect. These results are most readily explained by postulating that antibody-dependent cell-mediated cytotoxicity is the mechanism of clonal depletion. However, antibody-dependent complement-mediated cytotoxicity may be involved. If it is, then either neonatal cells are more

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.

22 March 1974; revised 11 June 1974 ■

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