Atómico and Instituto de Física, located at San Carlos de Bariloche, Argentina, which were used as base for the operation led by Ake Nilsson-Vinterbäck, from Uppsala, then visiting professor at Bariloche. I. U. Olsson, of Uppsala, helped with the selection of the samples. The Argentine Dirección General de Parques Nacionales offered the assistance of the three Intendencias of Parque Nacional Los Alerces, Lanín and Nahuel Huapi. B. Arschanow of the last park joined some expeditions and analyzed the cores, and E. Rodríguez of the Administración General de Bosques, Buenos Aires, did the first ring counting of the sections, later recounted by M. Munaud (Louvain).

12. Felled with special authorization of the Argentine Dirección General de Parques Nacionales.
13. J. C. Vogel and H. T. Waterbolk, *Radiocarbon* **9**, 107 (1967).
14. J. C. Lerman, W. G. Mook, J. C. Vogel, in *Proc. Nobel Symp. 12th*, I. U. Olsen, Ed. (Almqvist, Uppsala; Wiley, New York, in press).
15. H. Godwin, *Nature* **195**, 984 (1962); E. S. Deevey, R. F. Flint, I. Rouse, *Radiocarbon* **5**, v (1963).
16. H. Craig, *J. Geol.* **62**, 115 (1954).
17. J. C. Lerman, W. G. Mook, J. C. Vogel, *Nature* **216**, 990 (1967).
18. A South African tree from Cape Town, which was also measured, differs less than 0.5 ± 1.5 per mil from the Patagonian trees. A smoothed conversion curve (*14*) drawn from the results of Fig. 1 was already used for estimating historical dates in samples from that continent (*13*, pp. 148–149).
19. We thank the persons mentioned and the staffs of the institutes that supported this investigation. Part of this research was done while J.C.L. belonged to the staff of the Bariloche institutes.
* Present address: Physics Institute, University of Bern, Bern, Switzerland.

29 April 1969

X-ray Resistant Cell Required for the Induction of in vitro Antibody Formation

Abstract. *Mouse spleen cells were separated into two populations on the basis of adherence to plastic. The recombination of these two populations was required for the in vitro antibody response to sheep erythrocytes. By separating the two populations from x-irradiated mice and combining each with the other population prepared from normal mice, it was demonstrated that the immunologic function of the nonadherent population only was highly sensitive to x-ray injury. In contrast, x-irradiation in doses as high as 1000 roentgens had no measurable effect on the immune function of adherent cells, that is, the population which first interacts with antigen and is composed principally of large phagocytic cells.*

The remarkable sensitivity of the immune response to x-ray (*1*) has been attributed to injury to macrophages, specifically to antigen "processing" by such cells (*2*). The present experiments, in which the in vitro response of mouse spleen cells to sheep erythrocytes was used (*3*), fail to support this suggestion. Spleen cells were separated into two different populations on the basis of their adherence to plastic (*4*). The adherent population, which comprises about 10 percent of the total cells, consists primarily of macrophages; the nonadherent population is composed primarily of small lymphoid cells. The recombination of both the adherent and nonadherent populations is required for the in vitro antibody response to sheep erythrocytes. Equally high in vitro responses are obtained whether sheep erythrocytes are added to the recombined populations and remain present during the 4 days of the response or are added only briefly to the adherent population. In this latter procedure, the adherent population is washed free of all detectable extracellular antigen before being combined with the nonadherent population; thus the first cell-antigen interaction is limited to the adherent population.

The following experiment demonstrated that the kinetics of suppression by x-ray was similar for the in vivo and in vitro responses. One group of mice was x-irradiated with doses of from 25 to 250 r and then immunized with sheep erythrocytes; a second group of similarly x-irradiated animals was killed and their spleen cells were immunized in vitro. After 4 days the number of spleen cells releasing hemolytic antibody (*5*) was measured in the living animals and also in the cultures (Table 1). When the radiosensitivity of the in vitro response was established, the two populations of cells separable in vitro were examined to determine the cell type impaired by x-ray. Two reciprocal experiments were performed: (i) adherent cells from x-irradiated mice were combined with nonadherent cells from normal mice and (ii) adherent cells of normal mice were combined with nonadherent cells from x-irradiated mice. These experiments demonstrated that the immunologic function of the nonadherent population alone was sensitive to x-irradiation.

In one such experiment, spleen cells were pooled from six female DBA/2J mice that had received 500 r total body x-irradiation 1 hour previously (*6*). The spleens were teased apart in Hanks balanced salt solution. Large cell aggregates, removed by 3-minute gravity sedimentation, were discarded. The suspension of remaining single cells was washed once, resuspended to a concentration of 1×10^7 cells per milliliter in modified Eagle's minimal essential medium with 10 percent fetal calf serum (MEM), and dispersed in 1.0-ml portions in Falcon plastic petri dishes (35 by 10 mm). The dishes were then incubated for 30 minutes (*7*). Nonadherent cells were removed from these dishes by aspiration, pooled, and reincubated in new petri dishes for 30 minutes to remove any remaining adherent cells. The nonadherent cells were again pooled, sedimented by centrifuga-

Table 1. X-ray suppression of the in vivo and in vitro plaque-forming cell response. Groups of mice were irradiated 1 hour before either being injected intravenously with 2×10^8 sheep erythrocytes or killed and their spleen cell suspensions immunized in vitro with the same antigen. The in vitro and in vivo response were measured 4 days later.

X-ray dose	Plaque-forming cells	
	In vivo*	In vitro†
None	155	145
25	99	58
50	46	42
100	36	12
175	8	3
250	2	0

* The number recorded × 400 is the number of plaque-forming cells per spleen. The number is the average count of six slides; the slides were duplicates from three mice. † The number recorded is plaque-forming cells for 10^6 spleen cells initially cultured per dish. The number is the average count of eight slides; the slides were duplicates from each of quadruplicate cultures.

Table 2. Plaque-forming cell response of various combinations of cell populations obtained from normal and x-irradiated mice. Adherent and nonadherent cell populations were obtained from mice 1 hour after irradiation with 500 r, or from normal mice. The plaque-forming cell response was measured at 4 days. Each number is the average of counts on six slides; the slides were duplicates from each of the triplicate cultures. In vivo controls included three mice receiving the same dose of x-ray and three normal mice; the six mice were injected intravenously with 2×10^8 sheep erythrocytes. Duplicate slide counts were made on each spleen 4 days after immunization. Spleens of x-irradiated mice contained 60 ± 49 plaque-forming cells; spleens of the control mice contained 56,400 ± 12,600 plaque-forming cells.

Combination	Plaque-forming cells
Normal adherent cells + normal nonadherent cells	197
Normal adherent cells + x-irradiated nonadherent cells	18
X-irradiated adherent cells + normal nonadherent cells	156
X-irradiated adherent cells + x-irradiated nonadherent cells	0
Normal adherent cells only	7
Normal nonadherent cells only	7
X-irradiated adherent cells only	0
X-irradiated nonadherent cells only	0
Normal spleen cells unseparated	170
X-irradiated spleen cells unseparated	0
Normal spleen cells unseparated (no antigen)	0
X-irradiated spleen cells unseparated (no antigen)	0

tion, and resuspended to a concentration of 1×10^7 cells per milliliter. The original dishes containing adherent cells were incubated with 1.0 ml of MEM containing 1×10^7 sheep erythrocytes. After 30 minutes the medium and suspended erythrocytes were removed by aspiration. The dishes containing the adherent cells with phagocytized or adherent sheep erythrocytes, or both, were then washed twice by adding 1.0 ml of MEM to dishes and aspirating the fluid. These procedures were carried out simultaneously with spleen cells from normal mice that had not been x-irradiated.

The nonadherent cells from x-irradiated mice were added in 1-ml portions to the dishes of adherent cells from normal mice, and the nonadherent cells from normal mice were added in similar volumes to adherent cells from x-irradiated mice. These cultures and appropriate control cultures were then incubated. Each day 0.05 ml of a "nutritional cocktail" and 0.05 ml of fetal calf serum were added to the dishes. At the end of 4 days the cells were scraped from the dish, agitated to disperse clumps, and the number of direct plaque-producing cells was assayed by the slide modification of the Jerne technique. The results (Table 2) show that x-ray impaired the immune function of the nonadherent population but had no measurable effect on the adherent population.

The same experimental design was used to examine the effects of the dose of x-irradiation and of the interval between x-irradiation and the time the animal was killed. In some experiments spleen cells were prepared from mice killed 1 hour after x-irradiation with doses of 250, 750, and 1000 r. In other experiments spleen cells were prepared from mice killed 6, 24, or 48 hours after receiving 500 r. In every experiment adherent cells from x-irradiated mice functioned as well as adherent cells from normal mice. In contrast, the function of nonadherent cells from x-irradiated mice, whether combined with adherent cells from normal or x-irradiated mice, was markedly impaired. The results were consistent for the range of x-ray doses and time intervals tested (see Table 3).

It is conceivable that x-ray injury to adherent cells might become manifest if the interval between initiation of culture and antigenic stimulation were prolonged. To examine this possibility, adherent cells were prepared from mice 2 days after x-irradiation with 550 r. The adherent cells were cultured for 2 days and then exposed briefly to antigen before normal nonadherent cells were added to them. Thus, the total elapsed time was 4 days between x-irradiation and antigenic stimulation of cultures. The usual high plaque-forming cell response was observed 4 days after in vitro immunization, the response being equivalent to that for in vitro antigen-stimulated control cultures of fresh adherent and nonadherent cells from normal mice.

Table 3. Summary of results for cell combinations obtained from x-irradiated and normal mice.

Adherent population	Nonadherent population	Plaque-forming cells
Normal	Normal	167 ± 18*
Normal	X-ray	3 ± 1.5
X-ray	Normal	151 ± 19
X-ray	X-ray	0 ± 0

* The plaque-forming cell response is the mean ± standard error of the mean of in vitro responses from 11 experiments and represents the results of counts on 66 or more slides. Spleen cells were obtained from mice receiving 250 to 1000 r, 1 to 48 hours after x-irradiation. Responses were measured 4 days after immunization.

These experiments show that the immune function of nonadherent cells is sensitive to x-irradiation, and conversely, that the immune function of adherent cells is highly resistant to x-ray injury. Other deductions can be made concerning nonadherent and adherent cells. First, nonadherent cells contain two subpopulations that are required for the in vitro response to sheep erythrocytes (*8*). Although the present experiments do not resolve whether one or the other (or both) of these subpopulations is sensitive to x-ray, the following two observations are relevant to this question. On the one hand, it is possible that neither subpopulation is directly injured by x-ray, but that the immune function is suppressed by some indirect mechanism such as release of a toxic product from injured cells. This seems unlikely since the plaque-forming cell response of 1×10^7 cells from x-irradiated mice was fully restored by addition of 8×10^6 nonadherent cells from normal mice. In other experiments from this laboratory, it has been found that thymocytes restore the in vitro antibody response of spleen cells from mice that have been thymectomized, heavily x-irradiated, and then given syngeneic bone-marrow cells (*9*). However, the addition in vitro of from 10^6 to 10^7 normal mouse thymus cells to cultures of unseparated spleen cells, or to cultures of separated and recombined spleen cells from x-irradiated mice, failed to restore the response. This result does not indicate whether the nonadherent population contains highly x-ray sensitive thymus-derived cells, but it does suggest a requirement for at least one x-ray sensitive subpopulation that cannot be substituted for by thymus cells. The other subpopulation in nonadherent cells probably consists of cells derived from bone marrow, cells that synthesize antibody and are identifiable as plaque-forming cells (*10*).

Second, adherent cells from the mouse spleen contain a subpopulation of 1 to 10 per 10,000 cells that are essential for the in vitro response to sheep erythrocytes (*8, 11*). The morphology and function of the cells in this subpopulation is undefined at present, but it is clear that their immunologic function is relatively resistant to x-irradiation. It seems unlikely, therefore, that these cells divide rapidly and repeatedly to give rise to antibody synthesizing (plaque-forming) cells. Adherent cells obtained from normal or x-irradiated mice (800 r, 24 to 72 hours previously) contain "rosette-forming cells." Rosettes are formed by the rapid and dense clustering of heterologous erythrocytes on the surface of about 1 per 10,000 adherent cells. Rosette-forming cells are different from background plaque-forming cells and are apparently specific for different non-cross-reacting heterologous erythrocytes. Thus, it is possible to demonstrate in adherent cells a subpopulation that interacts with erythrocyte antigens, but it has yet to be determined conclusively that this interaction is required for the in vivo or in vitro antibody response (*12*).

Most of the adherent cells are morphologically and functionally macrophages. It is not known whether they play any part in the immune response to sheep erythrocytes, but if they are functional, then that function is also resistant to x-irradiation.

Numerous investigators have suggested that ingestion and "processing" of antigen by macrophages is required for antibody synthesis by lymphoid cells (*13*). It has further been proposed that this phase of the immune response may be highly sensitive to x-irradiation. In the present experiments, the first antigen-cell interaction occurs with adherent cells only. Excess antigen is then removed so that the stimulus provided by antigen (directly or indirectly) to nonadherent cell populations is presumably only by interaction between adherent and nonadherent cells. If it is assumed that the adherent cells

necessary for the immune response are phagocytic and that these cells, by interacting with antigen, process antigen in some manner, then clearly x-irradiation does not injure macrophages primarily or any processing mechanism of these cells for sheep erythrocyte antigens.

Many elegant experiments in which cells are used to reconstitute heavily x-irradiated animals have identified radiosensitive populations required for the immune response, but it is obviously impossible to evaluate any requirement for radioresistant populations with such a model. This first demonstration, that a step or cell required for the antibody response is relatively x-ray resistant, may permit further dissection of the complex cell interactions required for the immune response.

JEFFREY ROSEMAN
Department of Pathology,
University of Chicago,
Chicago, Illinois 60615

References and Notes

1. F. J. Dixon, D. W. Talmage, P. H. Maurer, *J. Immunol.* **68**, 693 (1952). A good general evaluation of the effect of x-irradiation in the immune response is presented by W. H. Taliaferro, L. G. Taliaferro, B. N. Jaroslow [*Radiation and the Immune Mechanisms* (Academic Press, New York, 1964)]. The effect of x-irradiation on the mouse spleen plaque-forming cell response has been reported by J. C. Kennedy, J. E. Till, L. Siminovitch, E. A. McCulloch [*J. Immunol.* **94**, 715 (1965)].
2. R. Gallily and M. Feldman, *Immunology* **12**, 197 (1967); J. F. Pribnow and M. S. Silverman, *J. Immunol.* **98**, 225 (1967).
3. R. I. Mishell and R. W. Dutton, *Science* **153**, 1004 (1966); *J. Exp. Med.* **126**, 423 (1967).
4. D. E. Mosier, *Science* **158**, 1573 (1967).
5. All antibody responses are measured as the number of plaque-forming cells by using a slide modification (*2*) of the assay system described by N. K. Jerne and A. A. Nordin [*ibid.* **140**, 405 (1963)].
6. All x-irradiation was done with Maxitron General Electric 250 [250 kv(peak), 30 ma]. The mice, placed in Lusteroid tubes on a Lucite turntable (3½ turns per minute), were exposed to total body x-irradiation at a target distance of 78 cm (average output, 58.5 r/min) with 0.25 mm Cu and 1 mm Al filter (half-value layer, 1.05 mm Cu).
7. All incubations involved maintaining the cultures at 37°C on a rocker table at 13 oscillations per minute in an atmosphere of 7 percent O_2, 10 percent CO_2, and the balance N_2.
8. D. E. Mosier and L. Coppleson, *Proc. Nat. Acad. Sci. U.S.* **61**, 542 (1968).
9. D. E. Mosier, F. W. Fitch, D. A. Rowley, A. J. S. Davies, *Fed. Proc.* **28**, 375 (1969).
10. G. F. Mitchell and J. F. A. P. Miller, *J. Exp. Med.* **128**, 821 (1968).
11. Attempts to substitute other phagocytic cells (for example, mouse Kupffer cells or rat spleen adherent cells) for mouse spleen adherent cells have been unsuccessful.
12. D. A. Rowley, unpublished data.
13. M. Fishman, *Nature* **183**, 1200 (1959); —— and F. L. Adler, *J. Exp. Med.* **117**, 595 (1963); M. D. Schoengerg, V. R. Mumaw, R. D. Moore, A. S. Weisberger, *Science* **143**, 964 (1964); G. Harris, *J. Immunol.* **9**, 529 (1965).
14. Supported by PHS grants AI-96-08 and HE-05667, and the Argonne Cancer Research Hospital, operated by the University of Chicago for AEC. The advice of Drs. D. A. Rowley and F. W. Fitch, and the technical assistance of Miss Helga Tremmel, are sincerely acknowledged.

29 May 1969

Mammary Alveolar Epithelial Cells: Effect of Hydrocortisone on Ultrastructure

Abstract. *Hydrocortisone is necessary for the formation of rough endoplasmic reticulum in mammary alveolar epithelial cells. This membrane system is required for the synthesis of the milk protein, casein, but it is not required for the synthesis of a nonmilk protein fraction.*

Insulin, hydrocortisone, and prolactin are necessary for the induction of casein synthesis by mouse mammary gland explants (*1*). After 4 days of culture with insulin and hydrocortisone (*2*), explants from mice midway through pregnancy do not make detectable casein; however, within 12 hours after the addition of prolactin these explants do make casein. In contrast, explants that have been cultured in the absence of hydrocortisone (medium containing

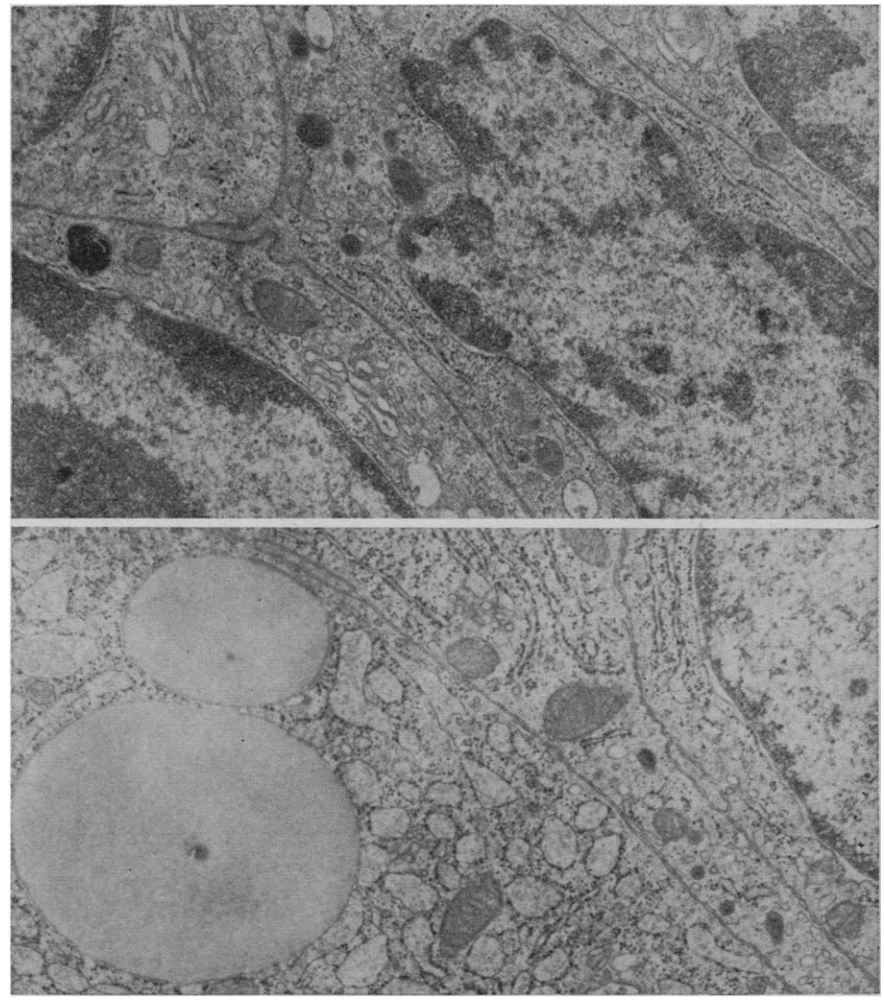

Fig. 1 (top). Ultrastructure of alveolar epithelium from mammary gland of C3H/HeN mouse midway through pregnancy. This micrograph shows portions of four alveolar epithelial cells from explants cultured for 96 hours in Medium 199 (*1*), containing 5 μg/ml of crystalline beef insulin (Lilly). The explants were fixed in Karnovsky's fixative (*7*) and embedded in Maraglas (*8*). Sections were cut on a Porter-Blum MT-1 ultramicrotome, stained with lead citrate and uranyl acetate (*9*), and examined in an RCA EMU-3G electron microscope. In most respects, these cells resemble the alveolar epithelial cells seen in the uncultured tissue. The cytoplasm contains very little rough endoplasmic reticulum. The epithelium contains no visible secretory products (approximately × 20,400). Fig. 2 (bottom). Ultrastructure of alveolar epithelium in explants derived from the same animals as in Fig. 1, cultured for 96 hours in Medium 199 containing 5 μg/ml of crystalline beef insulin (Lilly) and 5 μg/ml of hydrocortisone. The tissue was fixed and prepared as in Fig. 1. Rough endoplasmic reticulum (RER) is present throughout the cytoplasm. It often forms parallel nondilated cisternae and sometimes whorls. In some cells the RER is dilated. Lipid droplets (left side of micrograph) are often present in the cytoplasm (approximately × 21,250).