Demerara Plateau, like that beneath the Blake Plateau and Bahamas, had already been created by the late Jurassic, and that this crust provided a subsiding shallow-water platform upon which sediments were deposited.

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References and Notes

1. During the Cruise 9 of the U.S.N.S. *Kane* of United States Oceanographic Office.
2. C. D. Hollister and B. C. Heezen, *Science* **146**, 1573 (1964).
3. F. J. Pettijohn, *Sedimentary Rocks* (Harper, New York, ed. 2, 1957).
4. K. O. Emery, *U.S. Geol. Surv. Prof. Pap.* **529A**, 23 (1966); *Amer. Assoc. Petrol. Geol. Bull.* **52**, 445 (1968).
5. Paleontologic ages for fossiliferous carbonate material are reported by J. Antoine, W. Bryant, B. Jones [*Amer. Assoc. Petrol. Geol. Bull.* **51**, 257 (1967)] for West Florida Shelf; P. J. Fox, W. F. Ruddiman, W. B. F. Ryan, B. C. Heezen (*Tectonophysics*, in press) for Beata Ridge samples; P. J. Fox, E. Schreiber, B. C. Heezen [*Geol. Soc. Amer. Annu. Mtg. Abstr.* **7**, 69 (1969)] for Aves Ridge samples; B. C. Heezen and R. E. Sheridan [*Science* **154**, 1644 (1966)] for Blake Plateau; R. E. Sheridan, J. D. Smith, J. Gardner [*Amer. Assoc. Petrol. Geol. Bull.* **53**, 2551 (1969)] for Blake Plateau; M. Spencer [*ibid.* **51**, 263 (1967)] for Andros Island drill hole.
6. A. Wegener, *Origin of Continents and Oceans* (Dutton, New York, 1924).
7. G. O. Allard and V. J. Hurst, *Science* **163**, 528 (1969); G. O. Dickson, W. C. Pitman III, J. R. Heirtzler, *J. Geophys. Res.* **73**, 2087 (1968); P. M. Hurley, P. F. M. de Almeida, G. C. Melcher, U. G. Cordani, J. R. Rand, K. Kawashita, P. Vandoros, W. M. Pinson, Jr., H. W. Fairbairn, *Science* **157**, 495 (1967); A. E. Maxwell, R. P. Von Herzen, K. J. Hsü, J. E. Andrews, T. Saito, S. F. Percival, Jr., E. D. Milow, R. E. Boyce, *ibid.* **168**, 1047 (1970); D. A. Valencio and J. F. Vilas, *Nature* **223**, 1353 (1969); *ibid.* **225**, 262 (1970); J. B. Wright, *Tectonophysics* **6**, 301 (1968).
8. E. Bullard, J. E. Everett, J. G. Smith, *Phil. Trans. Roy. Soc. London Ser. H* **258**, 41 (1965).
9. Supported by grants from Office of Naval Research (N00014-67-A-0108-0004) and NSF (GA 10728). We gratefully acknowledge Drs. L. E. Stover and R. M. Jeffords of Esso Production Research Company who identified the fossils. We thank the officers, crew, and scientists of the U.S.N.S. *Kane* for their assistance during Cruise 9. Lamont-Doherty Geological Observatory Contribution No. 1609.

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Specificity of Antigen Recognition by Human Lymphocytes in vitro

Abstract. *Delayed hypersensitivity reactions in vivo are exquisitely specific, in terms of both a lack of response after induction of tolerance and a response after sensitization. These studies in vitro demonstrate that this specificity, at least at the level of antigen recognition, is probably derived from different populations of cells, each responding to different antigens.*

Lymphocytes from animals primed with certain antigens undergo blast transformation and mitosis in vitro when cultured with the priming antigen. An important question concerning this proliferation is whether the responding cells are part of a totipotent population in which any cell can respond to any antigen to which the animal is primed or whether the responding cells belong to distinct or partially distinct populations of antigen-specific cells.

Using their in vitro antibody plaque-forming-cell system, Dutton and Mishell have shown that different populations of antigen-specific precursor cells exist in the spleens of immunized mice (*1*). Addition of lethal amounts of tritiated thymidine to cultured cells dividing in response to sheep erythrocytes reduced the population to one which could no longer produce plaque-forming cells to the homologous antigen, but would produce plaque-forming cells in response to a heterologous antigen which does not cross-react with homologous antigen (burro erythrocytes). The production of plaque-forming cells to sheep erythrocytes in the suspension of mouse spleen cells in vitro probably involves the interaction of three distinct cell types. These include, in addition to adherent cells, nonadherent cells of two types, probably a "thymic" cell population and a "bone marrow" cell population, both of which may undergo proliferation in response to antigens (*2*). Which cell types show specificity in the Dutton-Mishell experiment is not clear.

One method of ascertaining whether the thymic cells show specificity is to use in vitro lymphocyte cultures of peripheral blood. This in vitro response appears to correlate with in vivo delayed hypersensitivity (cellular immunity) and thymic function rather than with humoral immunity (*3*). To determine whether specificity exists (that is, whether different cell populations respond to different soluble antigens) in this system similarly requires that one eliminate or inactivate all cells in culture that are responding to one antigen. If the hypothesis of separate cell populations is valid, then, while these cell cultures should no longer respond to the homologous antigen, one would expect a normal response to a heterologous antigen.

Inactivation of cells which are synthesizing DNA in response to a soluble antigen with the use of lethal amounts of tritiated thymidine is not accomplished in the leukocyte culture system where response is measured by tritiated thymidine incorporation. The incorporation of 5-bromodeoxyuridine (a thymidine analog) into DNA of dividing mammalian cells in culture provides an alternative means of inactivating specifically responding cells. Such cells become light-sensitive and are readily killed if exposed to light in the visible or near-visible region (*4*). We have adapted this approach to leukocyte cultures and present evidence suggesting that, in part, different cell populations respond to different soluble antigens.

Peripheral blood from healthy donors was drawn into heparinized syringes and allowed to settle for 2 to 3 hours. The leukocyte-rich plasma was removed, and leukocytes were obtained by centrifugation at 150*g* for 10 minutes. Cell-free plasma was prepared by two consecutive centrifugations of the supernatant plasma at 1650*g* for 10 minutes. Leukocytes were suspended and cultured in tissue culture medium 199, Earle's base (Gibco, Grand Island, New York) supplemented to contain approximate final concentrations of penicillin (100 unit/ml), streptomycin (100 μg/ml); and fresh or once-frozen cell-free plasma (20 ml/100 ml). Responding cells were suspended in a final concentration of 0.2×10^6 to 0.4×10^6 mononuclear cells per milliliter. Replicate 2-ml cultures were maintained for 7 days in a humidified atmosphere containing 5 percent CO_2. Cultures were labeled with 2 μc of tritiated thymidine (specific activity 1.9 c/mmole; Schwarz BioResearch) for 16 hours after 6 days of incubation and then harvested on day 7 with the addition of approximately 50 μg of nonradioactive thymidine, chilled on ice, and centrifuged at 1000*g* for 10 minutes. Incorporation of radioactive thymidine was assayed (*5*), and the average standard deviation of replicate cultures for these experiments was 20.9 percent.

Conditions for effective killing of cells synthesizing DNA with 5-bromo-

deoxyuridine (BUdR) and light treatment are described below. The BUdR (Sigma Chemical Company) was dissolved in Hanks balanced salt solution and added to each incubating cell culture for a period sufficient to insure its incorporation into cells synthesizing DNA. The cell cultures were exposed to visible or near-visible light by placing the open-bottom culture tube rack 3 to 5 cm above the two fluorescent lamps (15-watt, Champion "Cool White" tubes, 45 cm long) of an inverted desk lamp. Control cell cultures (nonilluminated) were placed near the lamp but shielded from the light. Excess BUdR was removed from cultures by centrifugation at 300*g* for 10 minutes and subsequent decantation of the culture medium. Cultures were vortexed once before the cells were suspended in fresh medium. The various culture stimulants used were streptokinase-streptodornase (Varidase) (SK-SD) No. 2201-66 (Lederle); tetanus toxoid, fluid (U.S.P. Eli Lilly); and mumps skin-test antigen (Eli Lilly).

The relation of stimulated cultures to sensitivity to BUdR and treatment with visible light are given in Figure 1. Cell cultures stimulated with mumps skin-test antigen (2.0 μl per milliliter of culture medium) were subjected to treatment with BUdR ($2.5 \times 10^{-6}M$) and light (90 minutes) and assayed on day 7. Other experiments showed that BUdR at higher concentrations ($10^{-3}M$ or $10^{-4}M$) showed an extensive cytolethal effect without illumination, although light somewhat increased this effect. At lower concentrations ($10^{-5}M$ to $10^{-6}M$) elimination of the response required illumination with light (a maximum effect was shown with 60 to 90 minutes of illumination), while at $10^{-7}M$ BUdR it was not possible to achieve complete elimination of the response. Treatment with BUdR and light during days 1 to 2 of culture has virtually no effect (Fig. 1). After 2 days, while the response for all cultures was somewhat depressed by the mechanics of the treatment procedure, only those cultures receiving both BUdR and illumination showed a further marked diminution. Thus cultures become sensitive to treatment with BUdR and light after 48 hours, and the response is virtually eliminated if cultures are treated after a 72-hour incubation period.

The results of an experiment designed to ascertain whether separate cell populations respond to different soluble antigens are given in Table 1.

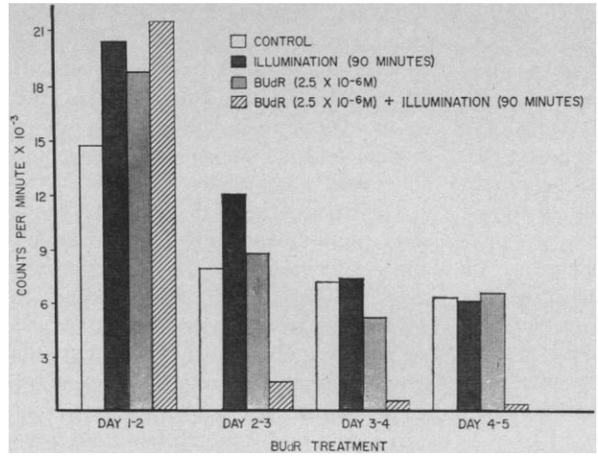

Fig. 1. The effect of BUdR and light treatment in eliminating the proliferative response of human lymphocytes to mumps skin-test antigen. The ordinate represents incorporation of tritiated thymidine in cultures responding to the antigen as assayed on day 7. The abscissa represents the days when cultures underwent treatment with BUdR and light. Each value represents the mean of duplicate cultures.

Multiple cell cultures from an individual sensitive to SK-SD, tetanus, and mumps antigens were stimulated with either SK-SD (25 unit/ml), mumps (1 μl/ml), or tetanus (1 μl/ml), or left unstimulated. After 48 hours of culture, BUdR ($2.5 \times 10^{-6}M$) was added to the appropriate cultures, and 24 hours later all cultures were illuminated for 1 hour. The process was repeated during the next 24 hours, so that after day 4 of culture appropriate cultures contained BUdR at a final concentration of $5.0 \times 10^{-6}M$, and all cultures had received 2 hours of exposure to visible light. (This double exposure over a 48-hour period is routine for these experiments.) Medium containing both the initial stimulant and excess BUdR was removed, and all cultures were replenished with medium containing either no antigen, the homologous antigen, or one of the heterologous antigens. Incubation was continued, and tritiated thymidine incorporation was assayed either on day 7 or day 10. The day 7 assays demonstrated that the cell cultures were responding to the initial stimulants and that the treatment with BUdR and light eliminated this response. Cultures that did not receive an initial stimulant but underwent BUdR-light treatment were able to respond well to each of the anti-

Table 1. Reciprocal restimulation with three soluble antigens in human leukocyte cultures after BUdR-light treatment. All cultures were illuminated for a total of 120 minutes. Results are expressed as mean counts per minute of triplicate cultures.

BUdR	Secondary stimulant	Day of assay	Radioactivity (count/min)
	No initial stimulant		
No	None	7	4,757
Yes	None	7	856
Yes	None	10	1,216
Yes	SK-SD (25 units/ml)	10	123,463
Yes	Tetanus (2 μl/ml)	10	32,324
Yes	Mumps (2 μl/ml)	10	16,058
	SK-SD (25 units/ml)		
No	None	7	101,048
Yes	None	7	2,048
Yes	None	10	953
Yes	SK-SD (25 units/ml)	10	2,937
Yes	Tetanus (2 μl/ml)	10	22,410
Yes	Mumps (2 μl/ml)	10	13,707
	Tetanus (1 μl/ml)		
No	None	7	68,584
Yes	None	7	1,507
Yes	None	10	1,241
Yes	SK-SD (25 units/ml)	10	177,389
Yes	Tetanus (2 μl/ml)	10	1,827
Yes	Mumps (2 μl/ml)	10	36,427
	Mumps (1 μl/ml)		
No	None	7	16,009
Yes	None	7	429
Yes	None	10	356
Yes	SK-SD (25 units/ml)	10	58,216
Yes	Tetanus (2 μl/ml)	10	41,453
Yes	Mumps (2 μl/ml)	10	205

gens as assayed on day 11. Cell cultures that initially responded to SK-SD and underwent BUdR-light treatment, could no longer respond significantly to SK-SD but did respond to tetanus and mumps antigens. Similarly, cultures that initially responded to tetanus or mumps and underwent BUdR-light treatment could no longer respond to the homologous antigens but did respond to the heterologous antigen. In each case it is important to compare the response of the secondary stimulant with the response at day 10 of cultures receiving an initial stimulant but no secondary stimulant.

The experiment presented in Table 1, showing reciprocal restimulation in all combinations between three soluble antigens, must be considered as strong evidence that, at least in part, different cells are committed to respond to different antigens, that is, the cells are not totipotent. As seen from the day 7 assays, the cells responded to soluble antigens given initially, and BUdR effectively eliminated this response. Such cultures after BUdR treatment could no longer respond significantly to the homologous antigen added at several concentrations but did respond to heterologous antigens. In those cultures that could be restimulated with heterologous antigens the response was sometimes significantly higher or lower than the response when that antigen was added to initially unstimulated cultures but treated with BUdR and light. Although cross-reactivity between the antigens, pluripotentiality of cells, or cell-bound potentiating factors would explain these findings, other possibilities exist.

Stimulation of a culture by optimum or nearly optimum concentrations of antigen has consistently allowed restimulation by heterologous antigens or phytohemagglutinin after BUdR treatment. The optimum stimulating dose of a soluble antigen was determined by maximum incorporation of tritiated thymidine on day 7 of culture. We have made 23 experiments testing reciprocal restimulation with five different soluble antigens and several cell donors. In 18 cases the restimulation was reciprocal (that is, the response to the homologous antigen is completely eliminated in both directions, and the cultures could still respond to the heterologous antigen). In the other five cases, after the response to the homologous antigen was eliminated, there was restimulation with the heterologous antigen in one direction, but no restimulation in the other direction. In no case have we found a culture that, after initially responding to one antigen and undergoing inactivation with BUdR, could still respond to the homologous antigen but not to a heterologous antigen to which the individual was sensitive.

Our data suggest that cells responding to soluble antigens in leukocyte cultures have specificity. Although complicated models could be proposed to invalidate this conclusion, it would seem very unlikely that responding cells are totipotent. Our results do not allow us to differentiate between a unipotent cell model and a pluripotent one; however, they do suggest that there is specificity in the "thymic" dependent cell population.

Leukocyte cultures are stimulated by a variety of substances. The use of BUdR to specifically kill only those cells that are synthesizing DNA in response to a stimulus should have considerable application in determining if further functional subpopulations exist among peripheral blood lymphocytes. For instance, one can test whether the cells that respond to nonspecific stimuli (such as phytochemagglutinin and pokeweed mitogen) include those cells that respond to specific stimuli (soluble antigens). This method should also be useful in investigating more complex cross-reacting antigen systems like those in mixed leukocyte cultures. The frequency of the initial responding unit is quite high, with minimum estimates in the range of 1 of 200 to 1 of 50 cells initially responding to a single allogeneic cell stimulus (*6*). The frequency of the initial responding unit in cultures stimulated by soluble antigens is not known. It will be interesting to determine whether there remain cells in a culture which are capable of responding to a second allogeneic cell stimulus after all the cells responding to the initial allogeneic cell stimulus have been eliminated by BUdR treatment.

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References and Notes

1. R. W. Dutton and R. I. Mishell, *Cold Spring Harbor Symp. Quant. Biol.* **32**, 407 (1967).
2. D. E. Mosier and L. W. Coppleson, *Proc. Nat. Acad. Sci. U.S.* **61**, 542 (1968); D. E. Mosier, F. W. Fitch, D. A. Rowley, A. J. S. Davis, *Nature* **225**, 276 (1970).
3. J. A. Mills, *J. Immunol.* **97**, 239 (1966); J. J. Oppenheim, *Fed. Proc.* **27**, 21 (1968).
4. B. Djordjevic and W. Szybalski, *J. Exp. Med.* **112**, 509 (19[illegible] T. T. Puck and F. Kao, *Proc. Nat. Acad. Sci. U.S.* **58**, 1227 (1967).
5. M. L. Bach, S. Solliday, M. Stambuk, in *Histocompatibility Testing*, P. Terasaki, Ed. (Munksgaard, Copenhagen, in press).
6. D. B. Wilson, J. L. Blyth, P. C. Nowell, *J. Exp. Med.* **128**, 1157 (19[illegible] F. H. Bach, H. Bock, K. Graupner, E. Day, H. Klostermann, *Proc. Nat. Acad. Sci. U.S.* **62**, 377 (1969).
7. We thank R. DeMars and M. L. Bach for suggestions and stimulating discussions. Supported by ONR grant N00014-67-A-0128-003, NIH grants GM15422 and AI08439, and NIH training grant GM00398. F.H.B. is an established investigator of the American Heart Association. This is paper No. 1412 from the Laboratory of Genetics, University of Wisconsin, Madison 53706.

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Cytotoxicity: Specificity after in vitro Sensitization

Abstract. *Animals sensitized in vivo against an allogeneic tissue subsequently show accelerated rejection specifically of that or antigenically similar tissues. Lymphocytes sensitized in vitro will destroy target cells isogeneic with the sensitizing cells. Lymphocytes sensitized in vitro can differentiate specifically between different allogeneic target cells—as occurs in vivo.*

The homograft response can be studied with in vitro models such as the mixed leukocyte culture (MLC) system (*1*, *2*) in which lymphocytes respond to foreign histocompatibility antigens on allogeneic cells, and target cell destruction assays in which aggressor (sensitized) leukocytes destroy allogeneic tissue (*3*). Extrapolation from in vitro models to the in vivo situation requires convincing evidence of a significant parallelism. We now report on the homograft response, in particular the specificity of tissue destruction after in vivo or in vitro sensitization.

An individual sensitized in vivo will show accelerated rejection of tissues from the sensitizing individual or tissues sharing antigens with the sensitizing tissue. That same individual will not, however, reject all grafts in an accelerated fashion and will not destroy autologous tissue. Likewise, sensitized leukocytes from that individual will, in vitro, preferentially kill target cells isogeneic to the sensitizing cells (*4*). In contrast, several reports suggested that leukocytes activated in vitro with a variety of agents—such as phytohemagglutinin, antigens to which the donor of the lymphocytes is sensitized, or those in MLC (*5*)—appear to have