References and Notes

1. E. J. DePraw, *DNA and Chromosomes* (Holt, Rinehart & Winston, New York, 1970).
2. D. E. Olins and A. L. Olins, *J. Cell Biol.* **57**, 715 (1972).
3. H. Ris and D. F. Kubai, *Annu. Rev. Genet.* **4**, 263 (1970).
4. V. Luzzati and A. Nicolaieff, *J. Mol. Biol.* **1**, 127 (1959); *ibid.* **7**, 142 (1963).
5. S. Bram and H. Ris, *ibid.* **55**, 325 (1971).
6. J. F. Pardon and M. H. F. Wilkins, *ibid.* **68**, 115 (1972).
7. J. G. Gall, *Chromosoma* **20**, 221 (1966); S. L. Wolfe, *J. Cell Biol.* **37**, 610 (1968); H. Ris, in *Handbook of Molecular Cytology*, A. Lima-de-Faria, Ed. (North-Holland, Amsterdam, 1969), p. 221; B. R. Zirkin, *J. Ultrastruct. Res.* **36**, 237 (1971); A. J. Solari, *Exp. Cell Res.* **67**, 161 (1971).
8. R. F. Itzaki and A. J. Rowe, *Biochim. Biophys. Acta* **186**, 158 (1969).
9. H. S. Slayter, T. Y. Shih, A. J. Adler, G. D. Fasman, *Biochemistry* **11**, 3044 (1972).
10. J. S. Kaye and R. McMaster-Kaye, *J. Cell Biol.* **31**, 159 (1969); H. G. Davies, *J. Cell Sci.* **3**, 129 (1968); A. C. Everid, J. V. Small, H. G. Davies, *ibid.* **7**, 35 (1970).
11. B. R. Zirkin and S.-K. Kim, *Exp. Cell Res.* **75**, 490 (1972).
12. F. Lampert, *Nature (Lond.)* **234**, 187 (1971).
13. V. G. Allfrey, V. C. Littau, A. E. Mirsky, *J. Cell Biol.* **21**, 213 (1964).
14. The buffer consisted of 0.05*M* sodium cacodylate, *p*H 7.5; 0.025*M* KCl; 0.005*M* $MgCl_2$; and 0.25*M* sucrose.
15. O. L. Miller, Jr., and B. R. Beatty, *Science* **164**, 955 (1969); *J. Cell Physiol.* **74** (Suppl. 1), 255 (1969); O. L. Miller, Jr., B. A. Hamkalo, C. A. Thomas, Jr., *Science* **169**, 392 (1970).
16. The Greek letter ν is suggested to denote these spheroid chromatin bodies. This notation was considered to be appropriate since they are both new and nucleohistone. Alternatively, we suggest that they be referred to as "deoxyribosomes" in order to emphasize their particular particulate analogy with ribosomes.
17. Solvents similar to these have been shown to extract ribonucleoprotein particles and nuclear sap proteins [H. Busch, *Histones and Other Nuclear Proteins* (Academic Press, New York, 1965)] including the RNA-containing interchromatin granules (*18, 19*). Furthermore, preliminary observations (D. E. Olins and E. B. Wright, unpublished) indicate that proteins extractable from chicken erythrocyte nuclei by washing in CKM buffer and 0.2*M* KCl contain no detectable histones, as judged by gel electrophoresis in buffers containing sodium dodecylsulfate [K. Weber and M. J. Osborn, *J. Biol. Chem.* **244**, 4406 (1969)].
18. A. Monneron and Y. Moulé, *Exp. Cell Res.* **51**, 531 (1968).
19. A. Monneron and W. Bernhard, *J. Ultrastruct. Res.* **27**, 266 (1969).
20. K. Brasch, V. L. Seligy, G. Setterfield, *Exp. Cell Res.* **65**, 61 (1971); A. L. Olins and D. E. Olins, unpublished observations.
21. R. A. Garrett, *Biochim. Biophys. Acta* **246**, 553 (1971).
22. O. L. Miller, Jr., and A. H. Bakken, *Acta Endocrinol. Suppl. 168* (1972), p. 155.
23. It is considered unlikely that the ν bodies and connecting strands described in this report might correspond to nuclear ribonucleoprotein particles. Interchromatin granules (200 to 250 Å in diameter) and perichromatin granules (400 to 450 Å in diameter) are considerably larger than the ν bodies (~70 Å in diameter) (*19*). Interchromatin granules would be expected to be extracted during the washes in CKM buffer (*18*). Furthermore, ν bodies are observed in considerably greater numbers than would be expected for the known ribonucleoprotein particles.
24. G. Zubay and P. Doty, *J. Mol. Biol.* **1**, 1 (1959).
25. J. H. Diggle and A. R. Peacocke, *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **18**, 138 (1971). From their data we take the following molecular weights: F2C, 20,800; F2B, 14,400; F2A2, 16,800; F2A1, 19,000; and F3, 13,000. The substitution of F1 histone for F2C in the rat tissue does not appreciably change the sum of molecular weights. Adding one molecule of each histone yields a total molecular weight of 84,000.
26. W. E. Hill, G. P. Rossetti, K. E. Van Holde, *J. Mol. Biol.* **44**, 263 (1969).

27. A preliminary report of this work was presented at the meeting of the American Society of Cell Biology, Miami, 15 November 1973. At the same meeting, C. L. F. Woodcock reported the observation of similar spherical particles in chromatin fibers. The authors thank O. L. Miller, Jr., and B. A. Hamkalo for advice and criticism and M. Hsie for excellent technical assistance. This work was sponsored, in part, by the Atomic Energy Commission under contract with Union Carbide Corporation, and, in part, by NIGMS grant 1 R01 GM 19334-01 to D.E.O. One of us (D.E.O.) is a recipient of NIGMS research career development award 5 K04 GM 40441-03.

19 July 1973

Tolerance to Heterologous Erythrocytes

Abstract. *Injection of a water-soluble nonantigenic fraction obtained from lysed sheep red blood cells virtually abolishes the subsequent immune response to the red cells. The suppression is systemic and appears to be serum mediated.*

Our understanding of the mechanisms underlying the production of immunological unresponsiveness or tolerance has increased over the past few years, but as yet there is not—and perhaps there cannot be—a unified theory of tolerance that would explain the data obtained from embryonic or neonatal transplantation tolerance experiments, from tumor enhancement and blocking work, from experiments involving disaggregated serum proteins, monomeric flagellin, or pneumococcal polysaccharides; nor may we be able to present a clear rationale for simultaneously understanding both low- and high-zone tolerance, both long-lasting and short-term tolerance, and both antigen- and antibody-mediated tolerance (*1*).

Tolerance to sheep red blood cells (SRBC) has been obtained both in neonatal and adult animals by a prolonged injection schedule with massive doses of SRBC (*2*). More generally, however, unresponsiveness has been induced by treatment with antigen in combination with cytotoxic agents such as cyclophosphamide (*3, 4*). Solubilized SRBC fractions have also been used in induction of tolerance (*5, 6*). There is no agreement concerning the basis for the induced tolerance to SRBC. Depending on the schedule of injections, the number of cells and the source of cells used in restitution experiments, and the timing of assessment of tolerance or of abrogation of tolerance, the lack of response to SRBC has been variously attributed to loss or inactivation of thymus cells, thymus-derived cells, bone marrow or bone marrow–derived cells, or to combinations of these cells (*4*). The possible functioning of suppressor cell populations has also been proposed (*4, 7*).

We have shown (*6*) that a supernatant fraction (after centrifugation at 40,000*g*) obtained after hypotonic lysis of SRBC contains material that can reduce to about 10 percent of normal the subsequent response of adult mice to SRBC. The tolerogenic material did not appear to be immunogenic as measured by induction of direct or indirect plaque-forming cells or by production of serum agglutinins or hemolysins. Reciprocal experiments with hemolyzate preparations of sheep and horse red blood cells indicated, moreover, that the tolerogenic effect of this supernatant is highly specific (*6*).

We now describe experiments designed to characterize the nature of the tolerance to SRBC induced by our supernatant fraction. The experiments show that this tolerance is not associated with a reduction or elimination of immunocompetent cells, but rather that it appears to be the result of a serum-mediated blocking effect.

Sheep red blood cells from individual sheep were purchased (ARS–Sprague-Dawley, Madison); they were washed before use. Tolerogenic preparations

Table 1. Ability of 10^7 spleen cells from normal or tolerant donors to respond to sheep red blood cells after injection into lethally irradiated normal or tolerant syngeneic host animals; PFC, plaque-forming cells; S.E., standard error.

Host	Donor	Experiments (No.)	Animals (No.)	PFC ± S.E. per spleen
Normal	Normal	3	15	4573 ± 882
	Tolerant		15	8878 ± 1666
Normal	Normal	3	13	2751 ± 796
Tolerant			18	402 ± 94

were obtained by hemolysis in hypotonic solution and centrifugation at a minimum of 34,000*g* for 200 minutes at 4°C (*6*). Tolerance was normally induced by five daily intraperitoneal injections of 0.4 ml of tolerogenic material. Each injection represented supernatant from approximately 2×10^8 SRBC. In the latest experiments, a single injection of 0.4 ml of a fivefold concentrate was used with equal success. After tolerogen treatment the response to SRBC was reduced to < 10 percent of that of the controls. C57BL/6 × DBA/2 (BDF_1) mice were used for all experiments. For restitution experiments host BDF_1 animals were irradiated with 850 roentgens total body irradiation (300 kv-peak, 20 ma, 0.5-mm Cu, 1-mm Al filtration; 70 r/min in air) a few hours prior to intravenous injection of test cell suspensions. The SRBC were injected intraperitoneally into these animals 24 hours later and again after 2 days, with plaque-forming cells being enumerated (*8*) on day 7 after irradiation.

Organ cultures were prepared (*9*), with 1-mm^2 full-thickness spleen explants placed in Millipore-filter well cultures in a medium consisting of Eagle's basal medium with 10 percent horse serum, 5 percent chick embryo extract, gentamycin (50 μg), and mycostatin (50 μg). The SRBC were added to the cultures which were then incubated at 37°C in a water-saturated atmosphere of 5 percent CO_2 in air. After 5 days, fragments were teased to obtain cells to be assayed for plaque-forming cells. Cell suspension cultures were prepared by the method of Click *et al.* (*10*). The mice were bled from the cervical region, the blood was permitted to clot at room temperature for 1 to 3 hours, the clotted blood was held overnight at 4°C, the clot was removed, and the remaining solution was centrifuged to obtain serum. The ability of the serum to influence the in vitro response to SRBC was assayed at a concentration of 20 μl per milliliter of medium.

Our initial assumption was that tolerance would be manifested either by thymus-derived (T) cells, bone marrow–derived (B) cells, or both. However, when we transferred spleen cells from tolerant animals into lethally irradiated host animals we found that these cells were fully capable of responding to SRBC; indeed the response was slightly enhanced (Table 1). In contrast, when we performed a reciprocal experiment, in which normal spleen cells were transferred into lethally irradiated, tolerogen-treated animals, the ability of these transferred normal spleen cells to respond to SRBC was significantly reduced (Table 1).

Table 2. Cell and organ culture studies with spleens and serum from mice made tolerant to sheep red blood cells; PFC, plaque-forming cells; MS, mouse serum.

Spleen cultures	Cultures (No.)	PFC ± S.E. per culture
Cell		
Normal	28	880.7 ± 140.8
Tolerant	25	800.0 ± 205.6
Organ		
Normal	51	61.6 ± 11.1
Tolerant	60	80.3 ± 12.7
Organ (normal)		
Standard medium	34	82.8 ± 17.0
Standard medium + 2% normal MS*		45.1 ± 12.4
Standard medium + 2% tolerant MS*	36	2.6 ± 0.6

* Four separate pools of serum.

We next turned to in vitro systems of assessing immune competence. Spleens from normal or tolerant animals were either cut into small fragments and grown in organ culture or prepared as a cell suspension and grown in stationary cell culture; the results were entirely comparable for the two systems (Table 2). Spleens from tolerant animals were fully competent to respond to SRBC in vitro.

Since these experiments all indicated that tolerant animals were unresponsive to SRBC not because of any inherent inability of their spleen cells to respond to this antigen but rather to some systemic blocking, we tested the effect of serum from normal or tolerant animals on the normal in vitro response to SRBC. The results (Table 2) show that serum from animals made tolerant to SRBC significantly reduces the in vitro immune response to this antigen.

Our experiments indicate that serum-mediated blocking activity is induced by injection of tolerogen, but they do not yet permit any conclusions concerning the nature of that inhibition. A number of ancillary observations (*6*, *11*) suggest that several simple interpretations are unlikely. (i) The tolerogen is nonantigenic as measured by both titration of serum agglutinin and hemolysin levels and by induction of plaque-forming cells in spleen, hence tolerance cannot be due to a standard antibody to SRBC (*6*); (ii) tolerance is long lasting in that the response to SRBC remains fully suppressed for at least 6 months (*11*); (iii) tolerance to SRBC is highly specific, as shown by reciprocal experiments involving sheep and horse red blood cells (*6*); (iv) the presence of native tolerogen itself does not significantly diminish the in vitro response to SRBC (*11*); and (v) serum blocking activity does not appear to be diminished by prior incubation of the serum with either SRBC or tolerogen (*11*).

It is well to consider that the sheep red blood cell is a cell, even if currently it is more generally thought of as an antigen. For this reason one might propose that soluble components of various tissue cells could act as tolerogens (*5*, *12*), and that the loss of ability to respond to such tolerogens might be prerequisite to the onset of autoimmune phenomena. The similarity between serum tolerance to SRBC and serum blocking activity associated with the presence of tumors is intriguing (*13*). It is now important to characterize the tolerogenic material, to identify the particular serum components necessary to achieve the unresponsive state, and to determine the mechanism by which the animal's immune response can be so specifically and so effectively suppressed.

ROBERT AUERBACH
JOAN ROETHLE
Department of Zoology, University of Wisconsin, Madison 53706

References and Notes

1. S. Leskowitz, *Annu. Rev. Microbiol.* **11**, 157 (1967); M. Landy and W. Braun, Eds., *Immunological Tolerance* (Academic Press, New York, 1969); U. Hopf, *Klin. Wochenschr.* **49**, 177 (1971); G. J. V. Nossal, in *Progress in Immunology*, B. Amos, Ed. (Academic Press, New York, 1971), p. 665; W. O. Weigle, J. M. Chiller, G. S. Habicht, *Transpl. Rev.* **8**, 3 (1972); J. G. Howard, *ibid.*, p. 50; in *Cell Differentiation*, R. Harris, P. Allin, and D. Viza, Eds. (Munskgaard, Copenhagen, 1973); E. Diener and M. Feldmann, *Transpl. Rev.* **8**, 76 (1972); L. Brent and M. E. French, chairmen, "1973 Workshop on mechanisms of tolerance and enhancement," *Transpl. Proc.* **5**, 1001 (1973); E. Diener, *Handbuch der Allgemeinen Pathologie* **7** (No. 3), 250 (1970).
2. H. Friedman, *Nature* **205**, 508 (1965); in *Immunological Tolerance*, M. Landy and W. Braun, Eds. (Academic Press, New York, 1969), p. 31; P. J. McCullagh, *Aust. J. Exp. Biol. Med. Sci.* **48**, 537 (1970).
3. A. W. Frish and G. H. Davis, *J. Lab. Clin. Med.* **68**, 103 (1966); A. C. Aisenberg, *J. Exp. Med.* **125**, 833 (1967); M. F. Dietrich and P. Dukor, *Pathol. Microbiol.* **30**, 309 (1967); L. H. J. Playfair, *Nature* **222**, 882 (1969); A. Many and R. S. Schwartz, *Proc. Soc. Exp. Biol. Med.* **133**, 754 (1970); R. K. Gershon, V. Wallis, A. J. S. Davies, E. Leuchars, *Nature* **218**, 381 (1968); J. Marbrook and B. C. Baguley, *Int. Arch. Allergy Appl. Immunol.* **41**, 802 (19 → J. F. A. P. Miller and G. F. Mitchell, *J. Exp. Med.* **131**, 675 (1970); A. C. Aisenberg and C. Murray, *Cell. Immunol.* **7**, 143 (1973).
4. K. D. Gershon and K. Kondo, *Immunology* **21**, 903 (1971).
5. J. Palmer, *Annu. Rep. Hall Inst., 1970–71*

(1972); P. Fetherstonhaugh, *Int. Arch. Allergy Appl. Immunol.* **39**, 310 (1970); C. R. Parish, *Eur. J. Immunol.* **2**, 143 (1972).
6. T. H. Anderson, J. Roethle, R. Auerbach, *J. Exp. Med.* **136**, 1666 (1972).
7. R. W. Dutton, *ibid.* p. 1445.
8. J. A. Cunningham and A. Szenberg, *Immunology* **14**, 599 (1968).
9. A. Globerson and R. Auerbach, *J. Exp. Med.* **124**, 1001 (1966).
10. R. E. Click, L. Benck, B. J. Alter, *Cell. Immunol.* **3**, 156 (1972).
11. R. Auerbach, in *Cellular Selection and Regulation in the Immune Response* (Raven, New York, in press).
12. C. F. C. MacPherson and S.-L. Yo, *J. Immunol.* **110**, 1371 (1973).
13. K. E. Hellstrom and I. Hellstrom, *Annu. Rev. Microbiol.* **24**, 1371 (1973).
14. We thank S. Friedman, L. Kubai, M. Sulman, and C. Wilson for help in carrying out these experiments. Supported by NSF grant GB 36767 and NIH grant CA 13548.

24 August 1973

Detection of Heat-Labile Escherichia coli Enterotoxin with the Use of Adrenal Cells in Tissue Culture

Abstract. *Cell-free culture filtrates of heat-labile enterotoxin-producing strains of* Escherichia coli *are capable of inducing morphological changes and steroidogenesis in monolayer cultures of adrenal cells. These tissue culture changes are similar to those induced by cholera enterotoxin and cannot be effected by culture filtrates of other enterotoxigenic or enteropathogenic types of bacteria. The results of the tissue culture studies correlated well with those done in the standard intestinal-loop systems and suggest that this tissue culture system could be used to significantly aid epidemiological and molecular studies with heat-labile* Escherichia coli *enterotoxin.*

The toxic manifestations of cholera enterotoxin and crude cell-free extracts of enteropathogenic strains of *Escherichia coli* have been shown to be associated with activation of intestinal mucosal adenyl cyclase and increased intracellular levels of cyclic adenosine monophosphate (AMP) (*1*). Several extraintestinal effects of cholera enterotoxin are also thought to be linked to stimulation of the adenyl cyclase–cyclic AMP system (*2*). Recently Donta *et al.* demonstrated that picogram quantities of the purified cholera enterotoxin were capable of inducing morphologic changes and steroidogenesis in monolayer cultures of adrenal cells in tissue culture (*3*). We report here that cell-free culture filtrates from enterotoxigenic strains of *E. coli*, but not those from nontoxigenic strains, are capable of inducing morphological changes and steroidogenesis similar to those induced by cholera enterotoxin.

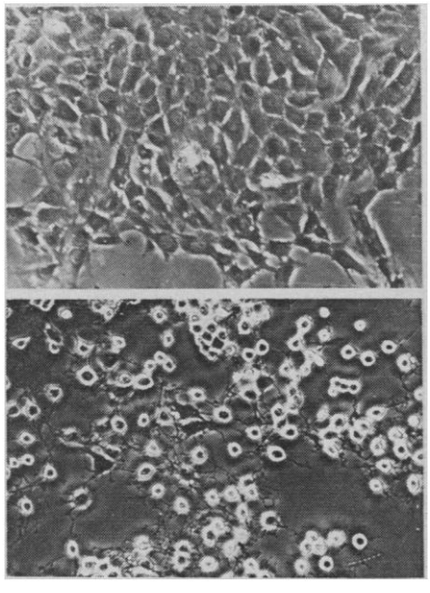

Fig. 1. Photomicrographs (phase-contrast, unstained, ×100) of monolayer cultures of Y1 cells. (Top) Untreated cells. (Bottom) Y1 cells 20 hours after addition of 0.1 ml of a culture filtrate of *E. coli* 0148 to the tissue culture medium.

A variety of *E. coli* strains isolated from American soldiers in Vietnam with diarrhea were provided by Dr. Samuel Formal of the Walter Reed Army Institute of Medical Research. These lyophilized strains were grown in trypticase–soy broth at 37°C in a shaking water bath for 24 hours. Then the culture was centrifuged to remove the bacteria and the supernatant was filtered through 0.22-μm filters for use in the tissue culture or ileal-loop studies. For the tissue culture studies, 0.2 ml of the sterile filtrate was added to 2.0 ml of the tissue culture medium (Ham's nutrient mixture F-10 supplemented with 15 percent horse and 2.5 percent fetal calf serum) which bathed late-logarithmically growing monolayer cultures of Y1 adrenal cells on 60- by 15-mm disposable plates (Falcon), and the plates were incubated at 37°C in a humidified atmosphere of 95 percent air, 5 percent CO_2. Quantitation of the Δ^4,3-ketosteroids that were secreted into the tissue culture medium was done by extracting the steroids from the medium with methylene chloride, separating the aqueous and organic phases, and evaporating the latter to dryness, then redissolving the residue in absolute alcohol and determining the solution's absorption at 242 nm. The spectrophotometric values were then converted to and expressed as nanomoles of steroid per plate of cells (or milligrams of cell protein) per incubation time interval. The jejunal-loop studies were performed in triplicate in 9-week-old rabbits, with aliquots of the same sterile filtrates used for the tissue culture studies. Two milliliters of the filtrate was used for each 10-cm loop, and after 18 hours the resultant fluid accumulation was expressed as milliliters of fluid per centimeter of gut (*4*).

Figure 1 depicts the morphological changes that are inducible by enterotoxigenic strains of *E. coli* and which closely resemble those effected by adrenocorticotropin (ACTH) and cholera enterotoxin. Whereas the changes inducible by ACTH begin a few min-

Table 1. Comparison of the responses of Y1 adrenal cells (morphological changes and steroidogenesis) and 9-week-old rabbit jejunal loops to cell-free culture filtrates of *E. coli*. The jejunal-loop activity is expressed as milliliters of liquid per centimeter of intestinal loop and represents the average value of three 10-cm loops. Steroidogenesis is expressed as nanomoles of steroid produced per plate of cells per 24 hours of incubation (*3*); morphological changes are graded as those strongly positive (about 90 percent or more of cells rounded) at 4 to 6 hours (++) or those moderately positive (about 25 to 75 percent of all cells rounded) at 6 to 8 hours (+).

E. coli strain	Morphological changes	Jejunal-loop index	Steroidogenesis
0148	++	3.07	20.44
M42-43*	0	0	0
121 V2B	0	0	0
301V-2MC-334a	0	0	0
201V-3MC-3342	0	0	0
111V-3MC-B2C	0	0	0
H-12808	0	0	0
V.N. 211VB	0	0	0
V.N. 161V1B	0	0	0
5 IV BB	+	1.80	13.94
5 IV AB	++	2.03	19.22
71 IV 1B	+	0.77	9.38
145 IV-4MC	0	0	0
76 IV 2M	0	0	0
78 IV 2MC	0	0	0
98 1 1MC	0	0	0
971 1MC	0	0	0
H-10407	++	3.70	17.68
171V1MC	0	0	0
29A1V1B	0	0	0
1461V-1B	0	0	0
TML-R66†	0	0	0
B2C	+	0.97	9.72
B7A	++	2.93	20.44

* This is actually a strain of *Shigella flexneri*, not *E. coli*. † Not *E. coli*, but a strain of *Salmonella typhimurium*.