Isolation of a T-Lymphotropic Retrovirus from a Patient at Risk for Acquired Immune Deficiency Syndrome (AIDS)

F. Barré-Sinoussi; J. C. Chermann; F. Rey; M. T. Nugeyre; S. Chamaret; J. Gruest; C. Dauguet; C. Axler-Blin; F. Vézinet-Brun; C. Rouzioux; W. Rozenbaum; L. Montagnier

Isolation of a T-Lymphotropic Retrovirus from a Patient at Risk for Acquired Immune Deficiency Syndrome (AIDS)

Abstract. *A retrovirus belonging to the family of recently discovered human T-cell leukemia viruses (HTLV), but clearly distinct from each previous isolate, has been isolated from a Caucasian patient with signs and symptoms that often precede the acquired immune deficiency syndrome (AIDS). This virus is a typical type-C RNA tumor virus, buds from the cell membrane, prefers magnesium for reverse transcriptase activity, and has an internal antigen (p25) similar to HTLV p24. Antibodies from serum of this patient react with proteins from viruses of the HTLV-I subgroup, but type-specific antisera to HTLV-I do not precipitate proteins of the new isolate. The virus from this patient has been transmitted into cord blood lymphocytes, and the virus produced by these cells is similar to the original isolate. From these studies it is concluded that this virus as well as the previous HTLV isolates belong to a general family of T-lymphotropic retroviruses that are horizontally transmitted in humans and may be involved in several pathological syndromes, including AIDS.*

The acquired immune deficiency syndrome (AIDS) has recently been recognized in several countries (1). The disease has been reported mainly in homosexual males with multiple partners, and epidemiological studies suggest horizontal transmission by sexual routes (2) as well as by intravenous drug administration (3), and blood transfusion (4). The pronounced depression of cellular immunity that occurs in patients with AIDS and the quantitative modifications of subpopulations of their T lymphocytes (5) suggest that T cells or a subset of T cells might be a preferential target for the putative infectious agent. Alternatively, these modifications may result from subsequent infections. The depressed cellular immunity may result in serious opportunistic infections in AIDS patients, many of whom develop Kaposi's sarcoma (1). However, a picture of persistent multiple lymphadenopathies has also been described in homosexual males (6) and infants (7) who may or may not develop AIDS (8). The histological aspect of such lymph nodes is that of reactive hyperplasia. Such cases may correspond to an early or a milder form of the disease. We report here the isolation of a novel retrovirus from a lymph node of a homosexual patient with multiple lymphadenopathies. The virus appears to be a member of the human T-cell leukemia virus (HTLV) family (9).

The retrovirus was propagated in cultures of T lymphocytes from a healthy adult donor and from umbilical cord blood of newborn humans. Viral core proteins were not immunologically related to the p24 and p19 proteins of subgroup I of HTLV (9). However, serum of the patient reacted strongly with surface antigen (or antigens) present on HTLV-I–infected cells. Moreover, the ionic requirements of the viral reverse transcriptase were close to that of HTLV. Re-

cently, a type-C retrovirus was also identified in T cells from a patient with hairy cell leukemia. Analysis of the proteins of this virus showed they were related to, but clearly different from, proteins of previous HTLV isolates (10). Moreover, recent studies of the nucleic acid sequences of this new virus show it is less than 10 percent homologous to the earlier HTLV isolates (11). This virus was called HTLV-II to distinguish it from all the earlier, highly related viruses termed
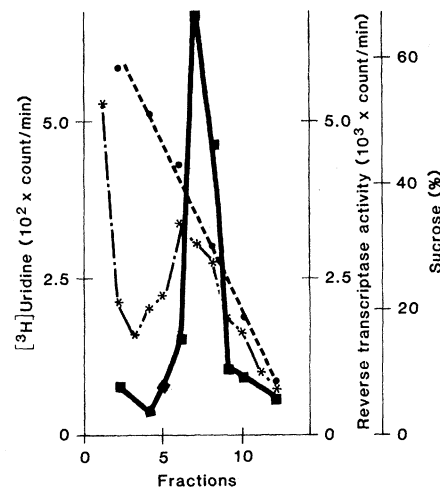

Fig. 1. Analysis of virus from patient 1 on sucrose gradients. Cord blood T lymphocytes infected with virus from patient 1 were labeled for 18 hours with [³H]uridine (28 Ci/mmole, Amersham; 20 μCi/ml). Cell-free supernatant was ultracentrifuged for 1 hour at 50,000 rev/min. The pellet was resuspended in 200 μl of NTE buffer (10 mM tris, pH 7.4, 100 mM NaCl, and 1 mM EDTA) and was centrifuged over a 3-ml linear sucrose gradient (10 to 60 percent) at 55,000 rev/min for 90 minutes in an IEC type SB 498 rotor. Fractions (200 μl) were collected, and 30 μl samples of each fraction were assayed for DNA polymerase activity with 5 mM Mg²⁺ and poly(A) · oligo-(dT)₁₂₋₁₈ as template primer; a 20-μl portion of each fraction was precipitated with 10 percent trichloroacetic acid and then filtered on a 0.45-μm Millipore filter. The ³H-labeled acid precipitable material was measured in a Packard β counter.

HTLV-I. The new retrovirus reported here appears to also differ from HTLV-II. We tentatively conclude that this virus, as well as all previous HTLV isolates, belong to a family of T-lymphotropic retroviruses that are horizontally transmitted in humans and may be involved in several pathological syndromes, including AIDS.

The patient was a 33-year-old homosexual male who sought medical consultation in December 1982 for cervical lymphadenopathy and asthenia (patient 1). Examination showed axillary and inguinal lymphadenopathies. Neither fever nor recent loss of weight were noted. The patient had a history of several episodes of gonorrhea and had been treated for syphilis in September 1982. During interviews he indicated that he had had more than 50 sexual partners per year and had traveled to many countries, including North Africa, Greece, and India. His last trip to New York was in 1979.

Laboratory tests indicated positive serology (immunoglobulin G) for cytomegalovirus (CMV) and Epstein-Barr virus. Herpes simplex virus was detected in cells from his throat that were cultured on human and monkey cells. A biopsy of a cervical lymph node was performed. One sample served for histological examination, which revealed follicular hyperplasia without change of the general architecture of the lymph node. Immunohistological studies revealed, in paracortical areas, numerous T lymphocytes (OKT3⁺). Typing of the whole cellular suspension indicated that 62 percent of the cells were T lymphocytes (OKT3⁺), 44 percent were T-helper cells (OKT4⁺), and 16 percent were suppressor cells (OKT8⁺).

Cells of the same biopsied lymph node were put in culture medium with phytohemagglutinin (PHA), T-cell growth factor (TCGF), and antiserum to human α interferon (12). The reason for using this antiserum was to neutralize endogenous interferon which is secreted by cells chronically infected by viruses, including retroviruses. In the mouse system, we had previously shown that antiserum to interferon could increase retrovirus production by a factor of 10 to 50 (13). After 3 days, the culture was continued in the same medium without PHA. Samples were regularly taken for assay of reverse transcriptase and for examination in the electron microscope.

After 15 days of culture, a reverse transcriptase activity was detected in the culture supernatant by using the ionic conditions described for HTLV-I (14). Virus production continued for 15 days

868

and decreased thereafter, in parallel with the decline of lymphocyte proliferation. Peripheral blood lymphocytes cultured in the same way were consistently negative for reverse transcriptase activity, even after 6 weeks. Cytomegalovirus could be detected, upon prolonged cocultivation with MRC5 cells, in the original biopsy tissue, but not in the cultured T lymphocytes at any time of the culture.

Virus transmission was attempted with the use of a culture of T lymphocytes established from an adult healthy donor of the Blood Transfusion Center at the Pasteur Institute. On day 3, half of the culture was cocultivated with lymphocytes from the biopsy after centrifugation of the mixed cell suspensions. Reverse transcriptase activity could be detected in the supernatant on day 15 of the coculture but was not detectable on days 5 and 10. The reverse transcriptase had the same characteristics as that released by the patient's cells and the amount released remained stable for 15 to 20 days. Cells of the uninfected culture of the donor lymphocytes did not release reverse transcriptase activity during this period or up to 6 weeks when the culture was discontinued.

The cell-free supernatant of the infected coculture was used to infect 3-day-old cultures of T lymphocytes from two umbilical cords, LC1 and LC5, in the presence of Polybrene (2 µg/ml). After a lag period of 7 days, a relatively high titer of reverse transcriptase activity was detected in both of the cord lymphocyte cultures. Identical cultures, which had not been infected, remained negative. These two successive infections clearly show that the virus could be propagated on normal lymphocytes from either newborns or adults.

That this new isolate was a retrovirus was further indicated by its density in a sucrose gradient, which was 1.16, and by its labeling with [^{3}H]uridine (Fig. 1). Electron microscopy of the infected umbilical cord lymphocytes showed characteristic immature particles with dense crescent (C-type) budding at the plasma membrane (Fig. 2).

Virus-infected cells from the original biopsy as well as infected lymphocytes from the first and second viral passages were used to determine the optimal requirements for reverse transcriptase activity and the template specificity of the enzyme. The results were the same in all instances. The reverse transcriptase activity displayed a strong affinity for poly(adenylate · oligodeoxythymidylate) [poly(A) · oligo(dT)], and required Mg^{2+} with an optimal concentration (5 mM) slightly lower than that for HTLV

(14) and an optimal pH of 7.8. The reaction was not inhibited by actinomycin D. This character, as well as the preferential specificity for riboseadenylate · deoxythymidylate over deoxyadenylate · deoxythymidylate, distinguish the viral enzyme from DNA-dependent polymerases.

We then determined whether or not this isolate was indistinguishable from HTLV-I isolates. Human T-cell leukemia virus has been isolated from cultured T lymphocytes of patients with T lymphomas and T leukemias [for a review, see (9)]. The antibodies used were specific for the p19 and p24 core proteins of HTLV-I. A monoclonal antibody to p19 (15) and a polyclonal goat antibody to p24 (16) were used in an indirect fluorescence assay against infected cells from the biopsy of patient 1 and lymphocytes obtained from a healthy donor and infected with the same virus. As shown in Table 1, the virus-producing cells did not react with either type of antibody, whereas two lines of cord lymphocytes chronically infected with HTLV (17) and used as controls showed strong surface fluorescence.

When serum from patient 1 was tested against infected lymphocytes from the biopsy the surface fluorescence was as

Table 1. Indirect immunofluorescence assay. Cells were washed with phosphate-buffered saline (PBS) and resuspended in the same buffer. Portions (5×10^4 cells) were spotted on slides, airdried and fixed for 10 minutes at room temperature in acetone. Slides were stored at $-80°C$ until use. Twenty microliters of either monoclonal antibody to HTLV p19 (diluted 1/400 in PBS) or goat antibody to HTLV p24 (diluted 1/400 in PBS) or serum from patient 1 diluted 1/10 in PBS was applied to cells and incubated for 45 minutes at 37°C. The appropriate fluorescein-conjugated antiserum (antiserum to mouse, goat, or human immunoglobulin G) was diluted and applied to the fixed cells for 30 minutes at room temperature. Slides were then washed three times in PBS. Cells were stained with Evans blue solution for 15 minutes and then washed extensively with water before microscopic examination.

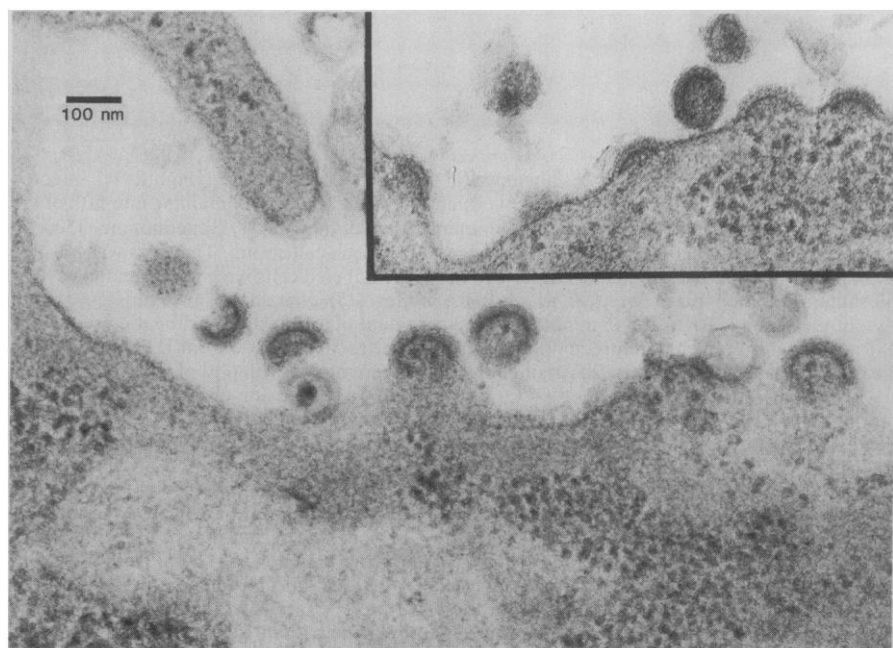| Cell type	Immunofluorescence (percent positive)		
	Antibody to p19	Antibody to p24	Serum from patient 1
Normal blood lymphocytes			
N 10916	−	−	−
LC$_1$	−	−	−
HTLV-producing cells			
C$_{91}$/PL	+ (90 to 100)	+ (90 to 100)	+ (90 to 100)
C$_{10}$/MJ$_2$	+ (90 to 100)	+ (90 to 100)	+ (90 to 100)
Virus-producing cells from			
Patient 1	−	−	+ (90 to 100)
LC$_1$/patient 1	−	−	± (0.5 to 2)
Patient 2	−	−	+ (90 to 100)

Fig. 2. Electron microscopy of thin sections of virus-producing cord lymphocytes. The inset shows various stages of particle budding at the cell surface.

intense as that of the control HTLV-producing lines. This suggests that serum of the patient contains antibodies that recognize a common antigen present on HTLV-I–producing cells and on the patient's lymphocytes. Similarly, cord lymphocytes infected with the virus from patient 1 did not react with antibodies to p19 or p24. Only a minor proportion of the cells (about 1 percent) reacted with the patient's serum. This may indicate that only this fraction of the cells was infected and produced virus. Alternatively, the antigen recognized by the patient's serum may contain cellular determinants that show less expression in T lymphocytes of newborns.

We also cultured T lymphocytes from a lymph node of another patient (patient 2) who presented with multiple adenopathies and had been in close contact with an AIDS case. These lymphocytes did not produce viral reverse transcriptase; however, they reacted in the immunofluorescence assay with serum from patient 1. Moreover, serum from patient 2 react-ed strongly with control HTLV-producing lines (not shown). In order to determine which viral antigen was recognized by antibodies present in the two patients' sera, several immunoprecipitation experiments were carried out. Cord lymphocytes infected with virus from patient 1 and uninfected controls were labeled with [^{35}S]methionine for 20 hours. Cells were lysed with detergents, and a cytoplasmic S10 extract was made. Labeled virus released in the supernatant was banded in a sucrose gradient. Both materials were immunoprecipitated by antiserum to HTLV-1 p24, by serum from patients 1 and 2, and by serum samples from healthy donors. Immunocomplexes were analyzed by polyacrylamide gel electrophoresis under denaturing conditions. Figure 3 shows that a p25 protein present in the virus-infected cells from patient 1 and in LC1 cells infected with this virus, was specifically recognized by serum from patients 1 and 2 but not by antiserum to HTLV-1 p24 or serum of normal donors. Conversely, the p24 present in control HTLV-infected cell extracts was recognized by antibodies to HTLV but not by serum from patient 1. A weak band (lane 2, Fig. 3B) could hardly be seen with serum from patient 2, suggesting some similarities of the p25 protein from this patient's cells with HTLV-1 p24. When purified, labeled virus from patient 1 was analyzed under similar conditions, three major proteins could be seen: the p25 protein and proteins with molecular weights of 80,000 and 45,000. The 45K protein may be due to contamination of the virus by cellular actin which was present in immunoprecipitates of all the cell extracts (Fig. 3).

These results, together with the immunofluorescence data, indicate that the retrovirus from patient 1 contains a major p25 protein, similar in size to that of HTLV-I but different immunologically. The DNA sequences of these and other members of the HTLV family are being compared. All attempts to infect other cells such as a B-lymphoblastoid cell line (Raji), immature or pre-T cell lines (CEM, HSB$_2$), and normal fibroblasts (feline and mink lung cell lines) were unsuccessful.

The role of this virus in the etiology of AIDS remains to be determined. Patient 1 had circulating antibodies against the virus, and some of the latter persisted in lymphocytes of his lymph node (or nodes). The virus-producing lymphocytes seemed to have no increased growth potential in vitro compared to the uninfected cells. Therefore, the multiple lymphadenopathies may represent a host reaction against the persistent viral infection rather than hyperproliferation of virus-infected lymphocytes. Other factors, such as repeated infection by the same virus or other bacterial and viral agents may, in some patients, overload this early defense mechanism and bring about an irreversible depletion of T cells involved in cellular immunity.

Fig. 3. Immunoprecipitation of ^{35}S-labeled viral proteins. Cord blood T-lymphocytes infected with virus from patient 1 were incubated overnight in culture medium containing one-fifth of the normal concentrations of methionine in minimum essential medium, [^{35}S]methionine (1500 Ci/mmole, Amersham; 50 μCi/ml), and 10 percent dialyzed fetal calf serum. The virus was purified by banding on a sucrose gradient as described in Fig. 1. Labeled cells were resuspended in 10 μl of saline and then lysed with 90 μl of RIPA buffer (*18*) containing aprotinin (500 U/ml; Zymofren, Specia) at 4°C for 15 minutes. The supernatant of a 10,000g centrifugation of the cell extract was used for immunoprecipitation. A similar extract was made from HTLV-producing C$_{91}$/PL cells (*17*). (A) Portions (20 μl) of cell extracts were mixed with 6 μl of serum, incubated for 2 hours at 37°C and overnight at +4°C. Then, 60 μl of a suspension of Protein A–Sepharose (10 mg/ml in RIPA buffer) were added. After 45 minutes of incubation at 4°C, immunocomplexes bound to Protein A–Sepharose were washed five times with RIPA buffer by centrifugation, heated for 3 minutes at 100°C in denaturing buffer and electrophoresed on 12.5 percent polyacrylamide-SDS slab gel (*19*). Lanes 1 to 5: Extract of LC$_1$ cells infected with virus from patient 1 and tested against 1, serum from patient 1; 2, serum from patient 2; 3, serum of a healthy donor; 4, goat antiserum to HTLV-Ip24; 5, normal goat serum. Lanes 6 to 10: C91/PL (HTLV-producing) cell extract tested with: 6, serum from patient 1; 7, serum from patient 2; serum of a healthy donor; 4, goat antiserum to HTLV-Ip24; 5, normal goat serum. (B) Portions (20 μl) of the band containing virus from patient 1 were treated with various antisera and processed as described for cell extracts. Lane 1, serum from patient 1; 2, serum from patient 2; 3, serum of a healthy donor; 4, serum of another healthy donor; 5, goat antiserum to HTLV-Ip24. Arrows indicate the p24–p25 protein. Molecular weights (in thousands) are indicated on the left.

F. BARRÉ-SINOUSSI, J. C. CHERMANN
F. REY, M. T. NUGEYRE
S. CHAMARET, J. GRUEST
C. DAUGUET, C. AXLER-BLIN
Institut Pasteur, Département de Virologie, 75724 Paris Cédex 15
F. VÉZINET-BRUN, C. ROUZIOUX
Hôpital Claude Bernard, Laboratoire Central—Virologie, 10 avenue de la Porte d'Aubervilliers, 75019 Paris
W. ROZENBAUM
Hôpital La Pitié-Salpétrière, Département de Santé Publique et Médecine Tropicale, 97 Boulevard de l'Hôpital, 75013 Paris
L. MONTAGNIER
Institut Pasteur, Departement de Virologie, 75724 Paris Cédex 15
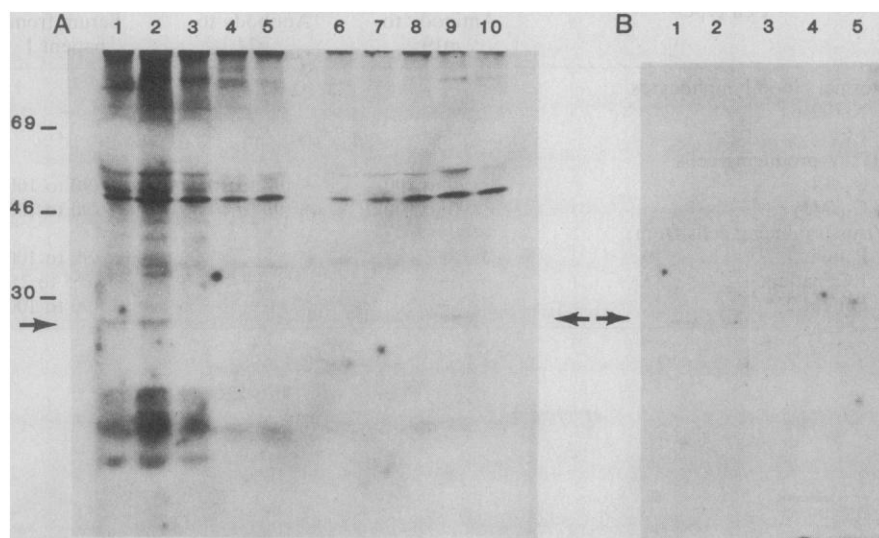
References and Notes

1. Centers for Disease Control, Task Force on Kaposi's Sarcoma and Opportunistic Infections, *N. Engl. J. Med.* **306**, 248 (1982).
2. M. Marmor *et al.*, *Lancet* **1982-II**, 1083 (1982); S. Fannin *et al.*, *Morbid. Mortal. Weekly Rep.* **31**, 305 (1982).
3. Centers for Disease Control, Task Force on Kaposi's Sarcoma and Opportunistic Infections, *Morbid. Mortal. Weekly Rep.* **31**, 507 (1982).
4. M. C. Poon *et al.*, *ibid.*, p. 644.
5. R. E. Stahl, A. Friedman-Kien, R. Dubin, M. Marmor, S. Zolla Parner, *Am. J. Med.* **73**, 171 (1982).
6. D. Mildvan *et al.*, *Morbid. Mortal. Weekly Rep.* **31**, 249 (1982).
7. R. O'Reilly *et al.*, *ibid.*, p. 665.
8. W. Rozenbaum *et al.*, *Lancet* **1982-II**, 572 (1982).
9. R. C. Gallo and M. S. Reitz, Jr., *J. Natl. Cancer Inst.* **69** (No. 6), 1209 (1982).
10. V. S. Kalyanaraman *et al.*, *Science* **218**, 571 (1982).
11. E. Gelmann, F. Wong-Staal, R. Gallo, personal communication.
12. The cells were grown in RPMI-1640 medium supplemented with antibiotics, $10^{-5}M$ β-mercaptoethanol, 10 percent fetal calf serum, 0.1 per-

cent sheep antibody to human α interferon (neutralizing titer, 6 IU at 10^{-5}), and 5 percent TCGF, free of PHA.
13. F. Barré-Sinoussi *et al.*, *Ann. Microbiol. (Inst. Pasteur)* **130B**, 349 (1979).
14. B. J. Poiesz *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7415 (1980).
15. M. Robert-Guroff *et al.*, *J. Exp. Med.* **154**, 1957 (1981).
16. V. S. Kalyanaraman *et al.*, *J. Virol.* **38**, 906 (1981).
17. M. Popovic, P. S. Sarin, M. Robert-Guroff, V. S. Kalyanaraman, D. Mann, J. Minowada, R. C. Gallo, *Science* **219**, 856 (1983).
18. R. E. Karess *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3154 (1979).
19. U. K. Laemmli, *Nature (London)* **227**, 608 (1970).
20. We thank Dr. Fradellizi for gifts of T-cell growth factor, R. C. Gallo for providing antibodies to HTLV and for HTLV-producing cells, Mrs. Le François for preparation of the cord lymphocytes, M. Lavergne (Institut Pasteur, Production) for gifts of fluorescein-conjugated antisera, F. Huraud for performing some of the HTLV tests, and members of the French Working Group on AIDS for helpful discussion.

19 April 1983

Heinz-Body Hemolytic Anemia from the Ingestion of Crude Oil: A Primary Toxic Effect in Marine Birds

Abstract. *Hemolytic anemia developed in young herring gulls and Atlantic puffins given daily oral doses of a Prudhoe Bay crude oil. Anemia developed 4 to 5 days after the initiation of oil ingestion and was accompanied by Heinz-body formation and a strong regenerative response. The data evince a toxic effect on circulating red blood cells involving an oxidative biochemical mechanism and the first clear evidence of a primary mechanism of toxicity from the ingestion of crude oil by birds.*

Petroleum oils regularly enter the marine environment through spills, runoff, and seepage (*1*). Large numbers of birds have died in association with marine oil spills (*2*), and the effects of oil on birds have been studied experimentally. Birds that become oiled ingest oil while preening (*3*), and oral doses of several petroleum oils have produced a wide range of sublethal toxic changes affecting growth, reproduction, osmoregulation, steroid metabolism, and hepatic function (*4–6*). Thus there is a firm basis for concern that oil pollution may produce subtle, sublethal effects in wild birds that impair reproduction or survival. This concern is heightened by the current emphasis on offshore oil development.

We report here that young herring gulls (*Larus argentatus*) and Atlantic puffins (*Fratercula arctica*) developed a severe hemolytic anemia after several days of oral dosing with a Prudhoe Bay crude oil (PBCO). Our data indicate that this was a primary toxic effect in which oxidative chemical processes damaged red blood cells in the peripheral circulation, and they constitute the first clear evidence of a primary toxic mechanism in experimental studies of the toxicity of ingested crude oil in birds.

In our initial experiments we used herring gull nestlings 2 to 3 weeks old,

taken from a coastal colony. These young birds adjust well to captivity and tolerate the manipulation required in laboratory work. Gulls were collected on Great Island, 50 km south of St. John's, Newfoundland, and held in pens at Memorial University of Newfoundland, St. John's (*7*). Pens were partially bedded with hay, and the birds were fed unlimited amounts of capelin (*Mallotus villosus*) and seawater. When all the birds were
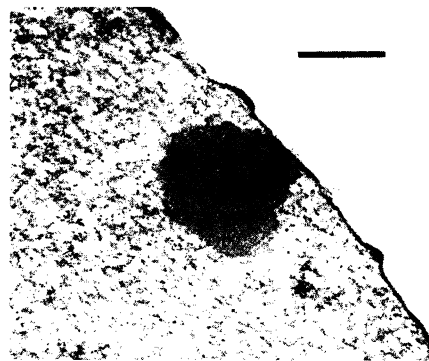

Fig. 1. Transmission electron micrograph of a Heinz body attached to the plasma membrane of a red blood cell from a herring gull which ingested 20 ml of Prudhoe Bay crude oil per kilogram per day for 4 days (experiment 1). This cell is a ghost erythrocyte, with most of the free hemoglobin lost from its cytoplasm; bar, 200×10^{-6} mm.

gaining weight (3 to 5 days after capture), they were divided into experimental groups of similar mean body weight (460 g) and weight distribution. Blood (1 ml) was taken from each bird, and experiments were begun immediately. In experiment 1, the birds were given either 10 or 20 ml of PBCO (*8*) per kilogram of body weight per day in gelatin capsules; controls received empty capsules. Dosing continued daily. Blood samples were drawn into heparinized tubes on day 5, and routine hematological measurements were made (*9*). Values for all groups prior to dosing and for controls on day 5 of dosing did not differ significantly. On day 5, blood taken from oil-dosed birds was dark brown and failed to redden on mixing with air; this result suggested a marked reduction in oxygen-carrying hemoglobin. The birds receiving oil were severely anemic, with packed cell volumes (PCV) reduced by 43 and 50 percent (Table 1). The plasma from these birds was rusty red, an indication of hemolysis either intravascularly or during sample handling. A strong regenerative response to the anemia was evident in the high reticulocyte count (Table 1). After the birds were killed, there was no evidence of trauma, enteric bleeding, or other hemorrhage. These data are sufficient to permit the classification of this anemia arising from oil ingestion as a hemolytic anemia (*10*).

Heinz bodies were abundant in erythrocytes from oil-dosed birds (Table 1). These were identified by two different staining techniques applied to fresh samples and in sections studied by light and transmission electron microscopy (Fig. 1) (*9*). Heinz bodies are dense granular masses in red cells thought to consist of precipitates of hemoglobin oxidized in the protein moiety. They are a classical feature of toxic hemolytic anemias produced by a variety of dissimilar chemicals linked mechanistically by the ability to cause destructive oxidative reactions in red cells (*11*). The presence of Heinz bodies is good evidence of a primary toxicosis of red cells and of an oxidative biochemical mechanism of toxicity. To probe other possible sites of destructive oxidative processes in these red cells, we measured methemoglobin, sulfhemoglobin, and reduced glutathione (GSH) in whole blood and extractable fluorescence (EF) in red cell membranes as an indicator of membrane lipid peroxidation (*12*). No sulfhemoglobin was detected. The range of methemoglobin values was wide in all groups, and experimental animals did not differ significantly from controls. Means and standard deviations for the percentages of methemoglobin