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Immunohistological demonstration of respiratory syncytial virus antigens in Paget disease of bone

(slow virus infection/immunoperoxidase staining/immunofluorescent staining/bone cells)

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ABSTRACT **Respiratory syncytial virus antisera have been found to produce a positive immunohistologic response in osteoclasts in bone sections or in cells cultured from Paget disease lesions in 12 out of 12 patients tested. These experiments were carefully controlled by several means. Use of experimentally infected cells served as positive controls. Adsorption of antisera on human bone powder and KB cells did not remove the specific immunologic stain, but adsorption of the antisera by the virus did. Negative results were also obtained in osteoclasts of patients with primary or secondary hyperparathyroidism. In addition, negative results in specimens of Paget disease were found with antisera to measles; parainfluenza 1, 2, and 3; influenza A, B and C; rubella; and herpes simplex. These results are consistent with the hypothesis that the nuclear and cytoplasmic inclusions in the osteoclasts of Paget disease are a result of viral activity.**

Paget disease of bone is a chronic disorder of unknown etiology that is characterized by many of the features found in patients affected by slow virus infections of the nervous system such as subacute sclerosing panencephalitis (1). These include (*i*) a long period of clinically inapparent disease, (*ii*) confinement of the disease to a single organ, (*iii*) absence of granulocytes, (*iv*) absence of fever, (*v*) presence of giant cells, and (*vi*) nuclear inclusions resembling viral nucleocapsids. In Paget disease many of the osteoclasts are considerably larger and contain more nuclei than those found in normal bone or in bone under the stimulation of high levels of parathyroid hormone (2). These cells also have been shown to be abnormal upon electron microscopic examination (3). Studies of bone biopsy specimens from more than 100 patients in five laboratories have revealed the presence of nuclear and occasionally cytoplasmic inclusions in osteoclasts but not in osteoblasts or osteocytes (4–7). The inclusions are tubular structures of 10- to 15-nm diameter and may be found in clusters, in paracrystalline array, or in random distribution. These structures have been demonstrated in a variable percentage of the osteoclasts of all of the patients thus far evaluated but not in the osteoclasts of normal subjects, or of patients with a wide variety of metabolic bone diseases such as primary hyperparathyroidism.

The morphologic characteristics of the nuclear and cytoplasmic inclusions suggested that they might represent viral nucleocapsids of the paramyxovirus or pneumovirus group (3, 7). Cells from Paget bone lesions were grown in culture to facilitate viral isolation (1, 8). Utilizing immunohistologic techniques to search for viral antigens, we now present evidence of respiratory syncytial virus (RSV) antigen in bone biopsy specimens and cells cultured from Paget bone lesions.

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MATERIALS AND METHODS

Bone Specimens. Material from diagnosed cases of Paget disease of bone was obtained at surgery or in the form of paraffin blocks from the pathology files of the Orthopaedic Hospital and the Los Angeles County/University of Southern California Medical Center, Los Angeles, CA. Bone biopsies containing large numbers of osteoclasts from two patients—one with primary hyperparathyroidism and one with renal osteodystrophy—were used as controls as were normal human surgical bone specimens and rabbit bone.

Cell Cultures. Bone cells were isolated from fresh tissue obtained at surgery by explanting into culture flasks as described (8). Bone was dissected aseptically to exclude cartilage, muscle, and periosteum. Marrow was rinsed out with sterile, buffered salt solution containing antibiotics. Finely minced bone (2 gm/15 ml) was incubated at 37°C in BGJ_b medium (Fitton–Jackson modification; GIBCO) with 10% (vol/vol) fetal calf serum, or the cells were dispersed for a total of 2 hr in Puck's saline solution containing 0.125% trypsin and 0.01% disodium versenate, which was replaced every 30 min. Cells were harvested by slow centrifugation (100 × *g*), and trypsin was removed by thorough washing. Cells were seeded at 2×10^4 cells per 60-mm plate in BGJ_b medium with 10% fetal calf serum. The medium was changed every 3–4 days.

Morphologic Techniques. For identification of the nuclear and cytoplasmic inclusions of Paget disease in pathologic surgical specimens, bone was fixed in ice-cold 5% (vol/vol) glutaraldehyde or in modified Bouin's solution (pH 7) (9). Thick sections (1 μm) were stained with toluidine blue, and portions containing osteoclasts were thin sectioned and examined in a Zeiss 9S or AE1 Corinth 500 electron microscope (8).

Cells in culture were monitored by observation in the phase microscope, and representative fields were photographed. Growth patterns and changes during cultivation were recorded. Cells to be examined for histological characteristics and ultrastructure were washed with buffer and fixed in glutaraldehyde or Bouins' solution. They were then scraped from the flask, pelleted, and embedded, and sections were prepared and examined in the electron microscope.

Antisera and Sera. For screening purposes, parainfluenza 3, mumps, RSV, and herpes simplex antisera were obtained from Flow Laboratories, McLean, VA, and measles and mumps convalescent human antisera were obtained from the Communicable Disease Center of the U.S. Department of Health and Human Services. The National Institues of Health supplied

Abbreviations: RSV, respiratory syncytial virus; FITC, fluorescein isothiocyanate conjugate.

research reference reagents distributed by the National Institute of Allergy and Infectious Diseases, including antisera to influenza A, A1, A2, B, and C; mumps (Enders strain); herpes simplex; parainfluenza 1, 2, and 3; RSV-1976 and RSV-1979 (Long strain); and measles (Edmonston strain).

A purified antiserum to RSV (549) was a gift of Calderon Howe (Louisiana State University Medical Center, New Orleans, LA) (10). The virus had been obtained from the American Type Culture Collection and maintained by passage in African green monkey kidney cells, Vero cell line, or human oral epidermoid carcinoma cells, KB cell line. Monolayers were infected with passage fluid and maintained for 1–5 days in 0.1% lactalbumin hydrolysate (GIBCO) containing 2% fetal calf serum. Antiserum was prepared to RSV grown in KB cells. After low-speed centrifugation of infected cells, virus was pelleted from the supernatant fluid by centrifugation for 120 min at 20,000 × *g* onto a cushion of 75% (wt/vol) sucrose. After dialysis of the virus-containing fraction against phosphate-buffered saline, rabbits were immunized by four weekly footpad injections of virus in complete Freund's adjuvant. The animals were bled 10 days after the last injection. The blood sera were inactivated 30 min at 56°C and exhaustively adsorbed with lyophilized Vero cells. Antisera to RSV neutralized 10–100 $TCID_{50}$ (tissue culture infectious dose, infecting half the cells) of virus to high titers. One vial of this antiserum RSV (549) was used in our earliest experiments. Subsequently, the antiserum was adsorbed on frozen and thawed KB cells in suspension and centrifuged, and the supernatant antiserum was used in all later experiments. In the final experiments, RSV (549) and the guinea pig RSV antisera (NIH) were also adsorbed on human bone powder.

Goat and guinea pig sera were obtained from Flow Laboratories, whereas rabbit sera were obtained from healthy laboratory animals; human sera were from normal volunteers and Paget disease patients.

Immunofluorescence. Fluorescent antibody staining of cell cultures employed the indirect method with fluorescein isothiocyanate-conjugated (FITC) IgG (11). FITC antisera to rabbit and guinea pig IgG were obtained from Cappel Laboratories, Cochranville, PA. Cultured cells were grown on glass coverslips, washed thoroughly with phosphate-buffered saline, dried, fixed for 30 min in acetone at 4°C, stored at −20°C, and rinsed with saline prior to staining. Antisera to the virus to be tested were diluted 1:20 with saline (containing 1% goat serum for bone sections) and applied to the coverslip for 30 min at room temperature. After the antisera were removed, the coverslips were washed for 5 min with three changes of saline. The FITC IgG of the species in which the antisera were made was diluted 1:20, applied, and incubated for 30 min at room temperature. The coverslips were washed thoroughly and mounted in 10% (wt/vol) glycerol, pH 9. The same method was used for staining deparaffinized Paget disease bone slides (12). To control for nonspecific fluorescence, in each experiment specimens were evaluated utilizing nonimmune animal serum alone (in the absence of each antiserum). Bone cells from patients without Paget disease or rabbit bone cells were also evaluated simultaneously. RSV (Long)-infected KB cells grown and fixed in an outside laboratory served as a positive control for the RSV studies. Slides were considered positive only if the fluorescence was unequivocal. Borderline fluorescence as compared with control slides was considered negative.

Immunoperoxidase. The three-layer immunoperoxidase technique was used on the deparaffinized bone sections to identify a tissue antigen histochemically by the sequential application of (*i*) specific antiserum, (*ii*) goat IgG to the animal source of the antiserum, (*iii*) soluble horseradish peroxidase–antihorseradish peroxidase complex, and (*iv*) 3,3′-diaminobenzidine and hydrogen peroxide (13, 14). Reagents were obtained from Cappel Laboratories. Antisera were diluted from 1:10 to 1:1000, usually 1:50 or 1:100 as shown in Table 2. Controls for nonspecific staining were carried out as indicated under immunofluorescent methods. RSV (Long)-infected KB cells grown and fixed in an outside laboratory again served as a control for the RSV studies.

RESULTS

Ultrastructure. Tubular structures 10–12 nm in diameter were occasionally found in the nuclei of cells cultured from three patients (Fig. 1) (8). None were found in the cytoplasm. These structures resembled the nucleocapsids of a pneumovirus (15) or a paramyxovirus and were similar but slightly smaller than those found in osteoclasts of bone biopsies, which usually ranged from 12–15 nm. No complete viruses were found budding from the surfaces of cells in any of the cultures by electron microscopy.

All bone specimens contained osteoclasts with typical 12 to 15-nm tubular structures in the nuclei as described (4). These osteoclasts also contained cytoplasmic inclusions composed of tubules or aggregations of tubules similar to those in the nuclei. At times disintegration of nuclei occurred, releasing the tubular structures into the cytoplasm.

Immunofluorescence. Because osteoclasts are fully differentiated cells, they did not survive or replicate in the long-term cultures. Therefore, only mononuclear cells were available for evaluation. Positive nuclear fluorescence was observed 4 out of 7 times with guinea pig (GP/NIH-1976) antiserum in cell cultures from two patients (Fig. 2*A*). Positive cytoplasmic flu-

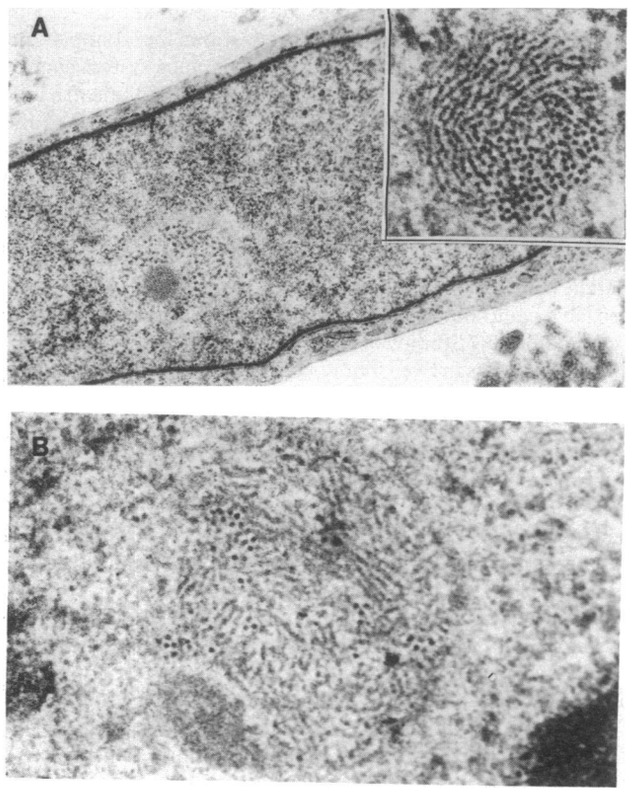

FIG. 1. (*A*) A cell cultured from bone of Paget disease patient 3, showing tubular structures morphologically similar to the nuclear inclusions found in Paget disease bone osteoclasts. (×12,000.) (*Inset*) Same nuclear inclusion. (×72,000.) (*B*) Nuclear inclusion in osteoclast from Paget disease bone lesion for comparison with the cell shown in *A*. (×72,000.)

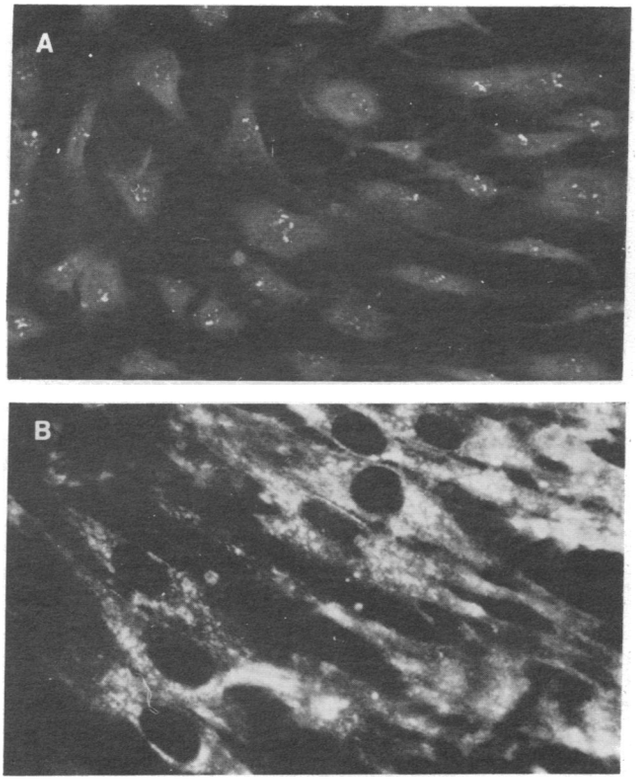

FIG. 2. (*A*) Cells cultured from bone of Paget disease patient 4 showing FITC-conjugated IgG labeling with RSV (GP/NIH) antiserum. Note fluorescence over nuclei of most cells. (×385.) (*B*) Cells cultured from bone of Paget disease patient 7 showing cytoplasmic fluorescence with RSV (549) antiserum. (×700.)

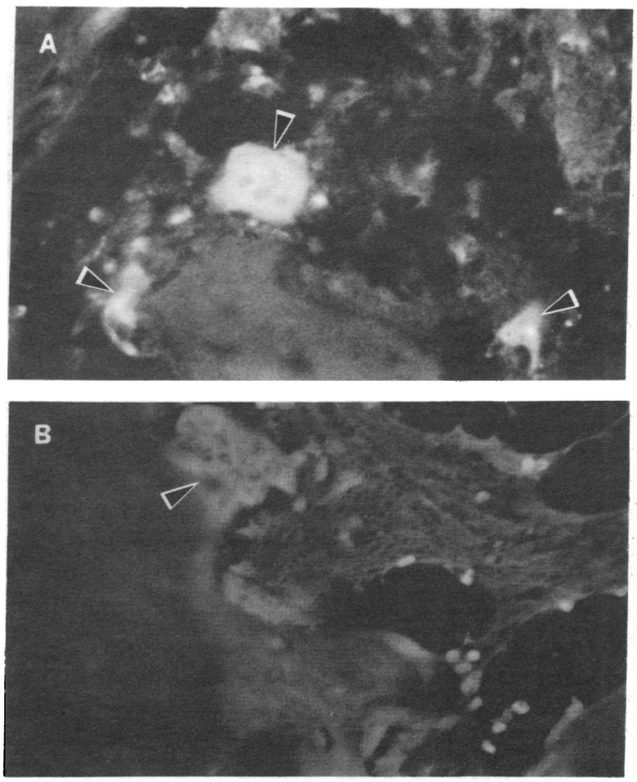

FIG. 3. (*A*) Bone of Paget disease patient 6 showing immunofluorescent staining of three osteoclasts with RSV (549) antiserum. (×260). (*B*) Bone of Paget disease patient 6 showing lack of fluorescence when normal rabbit serum was substituted for RSV (549) antiserum. (×260.)

orescence with rabbit or guinea pig RSV antisera was also found 17 times in cell cultures from seven patients (Table 1; Fig. 2*B*). In later experiments, osteoclasts in deparaffinized bone sections from four patients with Paget disease showed positive cytoplasmic fluorescence with rabbit RSV (549) antiserum adsorbed on KB cells and human bone powder and with guinea pig (GP/NIH-1979 and Flow Laboratories) antisera adsorbed on human bone powder (Table 1; Fig. 3 *A* and *B*).

Table 1. Immunofluorescent data from bone biopsies and bone cells cultured from patients with Paget disease by utilizing several RSV antisera*

	Anti-RSV antisera				
		549 absorbed on KB	GP/NIH		Flow
Substrate	549	cells	1976	1979	Labs.
Paget cells					
Patient 1	+1/1		−2/2		
Patient 2	+1/2		−1/1		
Patient 3†	+1/2	+1/1	+1/1 *nuc*		
Patient 4†	+1/3		+3/6 *nuc*		
Patient 5†		+1/1	−1/1		
Patient 6		+7/8			+1/1
Patient 7		+2/4‡		+1/3‡	
Paget bone					
Patient 6		+2/2‡			
Patient 10		+1/1‡		+1/1‡	
Patient 11		+1/1‡			+1/1‡
Patient 12		+1/1‡			+1/1‡

nuc, nuclear staining.

* Numerator is the number of positive or negative results obtained with each patient's specimen(s); the denominator is the number of separate experiments carried out.

† Cells in which nuclear inclusions were found by electron microscopy.

‡ Antiserum absorbed on human bone powder.

Antisera to measles; mumps; herpes simplex; parainfluenza 1, 2, and 3; rubella; and influenza A, A1, A2, and C and sera from five patients with Paget disease having high alkaline phosphatase values were all negative when tested against cells grown from several patients with Paget disease. Normal sera from three patients not having Paget disease were negative when tested against Paget disease bone cells. Normal rabbit bone cells tested negative with RSV, measles, and parainfluenza 3 antisera. RSV-infected KB cells showed positive cytoplasmic staining with RSV (549) antiserum adsorbed on KB cells.

Immunoperoxidase. Positive cytoplasmic staining with RSV (549) antiserum by the indirect immunoperoxidase system was obtained 27 times in osteoclasts present in deparaffinized bone sections from five patients with Paget disease (Table 2; Fig. 4 *A* and *B*). The antiserum was used at dilutions of 1:10 to 1:1000 (Fig. 5 *A* and *B*). Osteoclasts were considered positive only if the brown stain was clearly different from the background. Positive results were confirmed when read blind by three different investigators.

The control studies done on deparaffinized sections from the patients with primary hyperparathyroidism and renal osteodystrophy repeatedly showed no immunoperoxidase staining of osteoclasts with RSV (549) antiserum and with antisera to measles, rubella, parainfluenza 3, and herpes simplex. The cytoplasm of KB cells experimentally infected with RSV showed positive immunoperoxidase staining with RSV (549) antiserum adsorbed on KB cells. Antisera to rubella, measles, parainflu-

Table 2. Immunoperoxidase data obtained from bone biopsies of patients with Paget disease, primary hyperparathyroidism, and renal osteodystrophy, showing specificity of reaction to RSV antiserum

Substrate	Dilution of anti-serum	Anti-RSV (549) antiserum: Adsorbed on Vero cells	Adsorbed on KB cells	Adsorbed on KB cells plus RSV (Long)
Paget bone				
Patient 6	1:50		+4/4	−1/1
	1:100		+3/5	−4/4
	1:1000		+1/1	−1/1
Patient 8	1:10	+2/2	+1/1	
	1:50	+2/2	+2/2	
	1:100		+3/3	
	1:200		+1/1	
	1:500		+1/1	
Patient 9	1:50		+3/3	−2/2
Patient 10	1:50		+2/2*	
Patient 11	1:50		+2/2*	
Non-Paget bone				
Primary hyperparathyroidism	1:50		−4/4	−1/1
Renal osteodystrophy	1:50		−3/3	−3/3

* Antiserum adsorbed on human bone powder.

enza 3, and herpes simplex did not produce positive staining of osteoclasts in bone specimens from patients with Paget disease. Positive staining with mumps antiserum was found in the osteoclasts of two patients (6 and 8) in a total of four sections of 27 tested.

Adsorption of rabbit RSV (549) antiserum with RSV (Long) abolished the positive immunoperoxidase staining. This was demonstrated nine times on bone from two Paget disease patients (compare Fig. 5*A* with Fig. 5*C*).

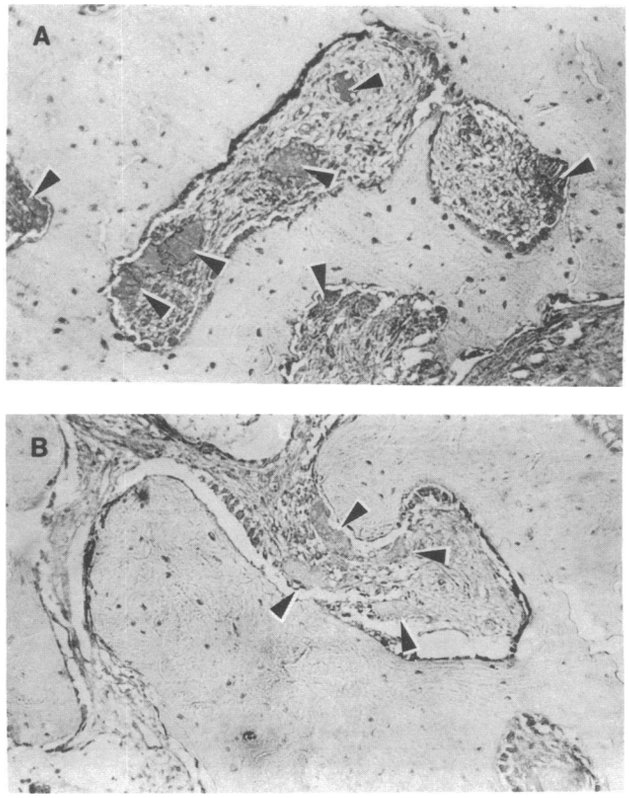

FIG. 4. (*A*) Bone of Paget disease patient 8 showing immunoperoxidase staining of several osteoclasts with RSV (549) antiserum (arrows). (×165.) (*B*) Bone of Paget disease patient 8 showing lack of immunoperoxidase staining of osteoclasts (arrows) when normal rabbit serum was substituted for RSV (549) antiserum. (×165.)

DISCUSSION

The results of this study support the hypothesis that the nuclear and cytoplasmic inclusions found in the osteoclasts of patients with Paget disease are the result of viral activity. In six of six bone specimens, positive staining of osteoclasts was found by

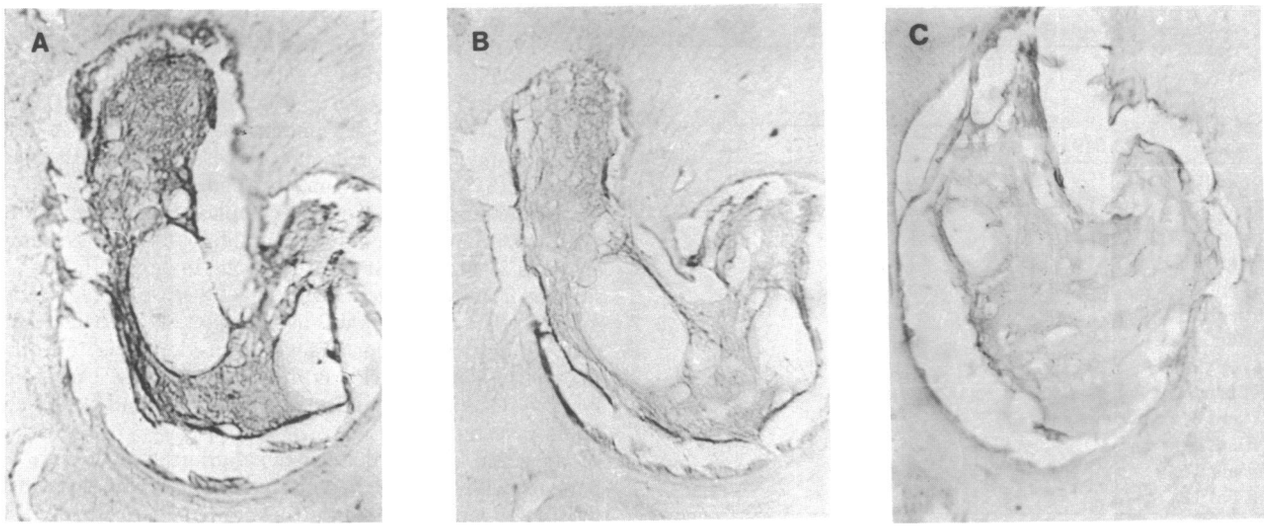

FIG. 5. (*A*) Resorption cavity in bone section from bone of Paget disease patient 6 nearly filled with two osteoclasts showing positive reaction to RSV (549) antiserum diluted 1:100. Note dark staining of cytoplasm of the cells. (×430.) (*B*) Serial section of the same resorption cavity as in *A*, treated with 1:1000 dilution of RSV (549) antiserum. Note staining is markedly reduced. (×430.) (*C*) Another resorption cavity (serial section) of same bone as in *A* following reaction with RSV (549) antiserum after the antiserum was absorbed with RSV (Long) virus. Note that at the same dilution (1:100), the virus has removed the positive staining.

using the immunoperoxidase technique or the immunofluorescent technique or both, with four RSV antisera. Osteoclasts do not survive in culture, but cells cultured from seven lesions of Paget bone disease also showed positive staining by using the technique of immunofluorescence. The specificity of the immunohistologic response was confirmed by several means. The positive staining was abolished after adsorbing the RSV (549) antiserum with RSV. Adsorption of the antiserum by KB cells, a human cell line derived from an oral carcinoma, produced no loss in staining characteristics. This was done because of the possibility that a nonspecific immune response might have been observed against KB antigens. Adsorption by human bone also failed to alter the staining characteristics. A critical finding was the absence of osteoclast staining in the bone sections of one patient with primary hyperparathyroidism and one with renal osteodystrophy. Human bone cells from patients without Paget disease and rabbit bone cells in culture also gave negative results. We conclude from these controlled studies that the osteoclasts of patients with Paget disease harbor an antigen(s) that is immunologically similar to an antigen(s) found in RSV.

RSV is an RNA virus of the pneumovirus group which is the most common etiologic factor in lower respiratory infections of children under 18 mo of age (16). The agent also is responsible for upper respiratory infections in adults. Isolation and identification of RSV have proven difficult because of the labile nature of the virus and its nucleocapsids (10). It is highly susceptible to freezing and thawing and will survive in cell culture only at a limited range of temperature.

In previous studies RSV antigens have been demonstrated primarily in the cytoplasm of infected cells in culture by immunofluorescent techniques (15, 17). We also have observed cytoplasmic immunofluorescence in RSV-infected KB cells. The osteoclasts of Paget disease and cells cultured from Paget bone lesions exhibited immunofluorescence and positive immunoperoxidase staining of the cytoplasm when evaluated with four antisera (Table 1 and 2). Because inclusions are always found in the nuclei of osteoclasts, the lack of nuclear staining with these antisera suggests that there are antigenic differences between the inclusions found in the nucleus and those in the cytoplasm. Levine found at least seven polypeptides in purified RSV (18). The largest were glycoproteins that may be associated with the viral envelope. One nucleocapsid-associated polypeptide and one lipoprotein of uncertain origin also were identified. Another RSV antiserum (GP/NIH-1976) we used did produce nuclear but not cytoplasmic immunofluorescence in cultured cells. This observation is compatible with a heterogeneity of antisera that label nuclear inclusions and cytoplasmic inclusions differently. Because the antisera we have used have not been completely characterized as to antigenic specificities, it is not possible to reach a conclusion concerning this hypothesis at present.

Although our data indicate that RSV antigens were present in the osteoclasts of all patients studied, it is possible that other viruses may be associated with Paget disease. Mumps antigen was demonstrated by immunoperoxidase staining in a small proportion of bone sections from two patients in whom RSV antigen was consistently found. In a preliminary report, an increased incidence of antibodies to RSV, mumps, and parainfluenza 3 in the serum of 20 patients with Paget disease was described (19). Basle *et al.* have reported the presence of measles-like antigen in the osteoclasts of Paget disease (20). Our own studies with different measles antisera were negative.

Cytoplasmic immunofluorescence was observed in the cultured cells despite the absence of nucleocapsid-like inclusions demonstrable by electron microscopy. There are two possible explanations for this observation. A defective viral genome may result in synthesis of the nucleocapsid antigen but failure of nucleocapsid assembly. Such a phenomenon has been described in HeLa cells infected with a temperature-sensitive mutant of RSV (16). The second possibility is that the cultured cells, which clearly are not osteoclasts, may fail to permit the full expression of the viral phenotype. This may be due to the failure of the host cell to allow production or proteolytic activation of some viral component required for assembly of the nucleocapsid (21, 22).

The association of nucleocapsid-like inclusions with immunohistochemical evidence of RSV in Paget disease has been demonstrated. The consistent finding of nuclear inclusions in the osteoclasts suggests that the agent may be a mutant of RSV (18) or an antigenically related pneumovirus or paramyxovirus.

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