DETECTION OF VIRAL INFECTION BY IMMUNOFLUORESCENCE IN FORMALIN-FIXED TISSUES, PRETREATED WITH TRYPSIN

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The presence of viral antigen in sections from formalin-fixed and paraffin-embedded human tissues was demonstrated by trypsin digestion followed by direct or indirect immunofluorescence. The specimens may be used for retrospective diagnosis.

The immunofluorescence technique has to be adapted to the suspected virus infection on the basis of previous histopathologic study. Variations of trypsin concentration time and temperature of incubation, expose different viral antigens and have to be previously tested for each unknown system. For measles virus detection in lung a stronger digestion has to be applied as compared to adenovirus or respiratory disease viruses in the same tissue. Flavivirus in liver tissue needs a weaker digestion. The reproducibility of the method makes it useful as a routine technique in diagnosis of virus infection.

Key words: virus diagnosis — immunofluorescence — retrospective diagnosis — trypsin digestion

Following the publication of the first paper by Huang et al. (1976) on the detection of hepatitis B by immunofluorescence in formalin-fixed and paraffin-embedded tissue sections after digestion with trypsin, the efficiency of this method has been confirmed by several authors. Diagnosis of rabies (Kennet et al., 1980) and of adenovirus (Chandler & Gorelkin, 1983) was obtained by direct and indirect immunofluorescence.

The technique has been modified and improved by the use of pepsin in addition to trypsin (Reed et al., 1983), by fixation with Bouin's fixative (Greenlee & Keeney, 1982) and by the use of other embedding media (Yoshisawa et al., 1977; Greenlee & Keeney, 1982).

To obtain a better histopathologic diagnosis in doubtful fatal cases of yellow fever, we employed trypsin digestion on 76 formalin-fixed and paraffin-embedded liver specimens with good results (Schatzmayr et al., 1984). Variations of trypsin concentration, time and temperature of exposure to the enzyme were tested.

Formalin-fixed and paraffin-embedded pulmonar tissues with giant cells containing measles virus were tested by this method and a good correlation with histopathology was obtained (Menasce, 1987). Trypsin digestion of fixed and embedded human organs is established at our laboratory as routine for retrospective studies of viral infections in pulmonar tissues, as well as for the detection of flavivirus in liver specimens.

MATERIAL AND METHODS

Tissues — Samples from autopsies of human cases were obtained from the Pathology laboratories of hospitals in Rio de Janeiro, Niterói and Belém. The tissues were fixed in 10% formalin and paraffin embedded. Histopathologic diagnosis was based on hematoxylin-eosin stained sections. Adjacent sections were employed for immunofluorescent assay. Some specimens were more than five years old.

Antisera — For herpes virus detection commercial herpes type 1 and type 2 FITC conjugate antisera were used. Serum from a monkey infected with yellow fever D-17 vaccinal strain virus was conjugated in our Department as described by Schatzmayr et al. (1984). Other antisera used, and their respective FITC conjugates, were obtained from different laboratories.

The sections were exposed to antisera and their conjugates for 30 min at 37°C in a humid chamber. Their dilutions in phosphate buffer saline (PBS) must be previously tested starting at 1:10. Usually a double or triple concentration of the antisera has to be used in paraffin embedded and trypsin digested tissues, as compared with the dilutions used for cell monolayers in tissue culture.

Methods — For both, direct and indirect immunofluorescence, Huang's et al. (1976) method of trypsin digestion was employed. Four micra thick sections were collected on glass slides previously coated with plastic glue diluted in equal parts of distilled water. They were left for 1 hour at 60°C and then immediately submitted to three washes of xylol, transferred to absolute ethanol and dehydrated by decreasing alcohol concentrations. After a quick washing in distilled water, the slides were transferred to a solution containing 0.1% trypsin and 0.1% $CaCl_2$ in PBS buffer, pH 7.8 adjusted with 0.1 N NaOH, for 2 hours at 37°C; trypsin concentration and time of incubation may vary in order to obtain a better digestion and to expose the specific virus antigens.

The slides were washed for 30 min in PBS, dried and incubated with antisera for 30 min at 37°C. If indirect immunofluorescence was used, after two washings in PBS, 5 min each, the conjugate was applied also for 30 min at 37°C, followed by two washes in PBS. After a short dip into distilled water, cover glasses were mounted with buffered glycerol (9 parts of glycerol and 1 part of PBS). The slides were examined in an epi-fluorescence Orthoplan-Leitz microscope equipped with a HBO — 150 W mercury lamp.

Controls — PBS and antisera to other than the expected virus were used as controls; a general nonspecific fluorescence of the tissues was observed. A counterstaining with Evans blue or naphthalene black did not always work well in this material. For antisera and conjugate control on single cells experimentally infected with a known virus, counterstaining with naphthalene black was useful.

For yellow fever control, hepatitis B orcein positive specimens were also included. Lung tissue with tuberculosis was used as an additional control for respiratory virus infection.

RESULTS

A total of 370 tests were performed in one or more tissues from 122 patients with the following results:

Adenovirus — Pulmonar tissues from 9 patients were examined for adenovirus antigen. Six of them had been positive by virus isolation in cell cultures and were also positive by trypsin-immunofluorescence (Figs. 1 and 2). The other three were negative by both methods. A two hour digestion with 0.1% trypsin gave better results than 0.25% trypsin; with the lower concentration, only one positive result was obtained. Previous treatment with 0.1% pepsin (1 hour) followed by 0.1% trypsin (1 hour) gave no better results. Without enzyme digestion, a general fluorescence was observed. The use of naphthalene black (0.1% in PBS) did not alter the results.

Measles virus — Measles virus antigen was detected by trypsin-immunofluorescence in giant cell-containing lung tissues of 9 out of 10 patients (Figs. 3 and 4). The best digestion was obtained by 0.25% trypsin for 2 hours. One case was positive in the left pulmonar lobe, while the right lobe was negative.

Dengue virus — Liver, spleen, lymph nodes and in one case muscle, heart and kidney tissues from 11 patients were tested for the presence of dengue virus type 1 antigen. Digestion was made by 0.1% trypsin. Strong fluorescence was obtained in one case with a flavivirus polyvalent hiperimmune ascitic fluid, obtained from CDC/Atlanta. By the use of a liver fragment kept at — 80°C, from the same patient, dengue virus type 1 was isolated by inoculation in *Aedes albopictus* cell line strain. The virus was reisolated from the original specimen by the same method, for confirmation. Monoclonal antibodies for dengue types 1 to 4, gave insatisfactory results. As control we used a anti-yellow fever monkey serum conjugate, which show negative result.

Respiratory disease virus — From five cases clinically suspected of respiratory syncytial virus infection, two were positive (Figs. 5 and 6), one was negative and two had doubtful results (weak immunofluorescence). Digestion was made with 0.25% trypsin but for one case also with 0.1% trypsin. The last test gave a better result, so the doubtful cases must be tested

with lower trypsin concentrations. Controls included anti-influenza, anti-parainfluenza, anti-measles and anti-adenovirus sera, all with negative immunofluorescence.

Two other cases of parainfluenza infection were tested. Trypsin concentrations was 0.1% and 0.25% with and without pre-treatment with 0.1% and 0.4% pepsin. While immunofluorescence was observed in one case treated with 0.1% trypsin alone or with previous 0.1% pepsin and 0.25% trypsin (Fig. 7), no immunofluorescence was detected when we use 0.4% pepsin. The other cases were all negative.

Herpes and cytomegalovirus — Liver and lung from three patients were examined with anti-herpes conjugates, and two of them were positive by immunofluorescence with a 0.25% trypsin digestion. The other case was negative. One case with CMV diagnosis was confirmed by immunofluorescence with 0.1% trypsin digestion.

DISCUSSION

Trypsin digestion is a crucial step during immunofluorescence diagnosis for virus infection of formalin-fixed and paraffin-embedded tissues. Keeping constant the temperature and time of digestion, starting at 0.1% of trypsin and increasing the enzyme concentration, different degrees of tissue and viral digestion are observed, so that specific antigens may be exposed or not. This was first demonstrated by Huang et al. (1976): hepatitis B surface antigen in liver cells is exposed after 2 hours or trypsin digestion, while it takes 4 hours to expose hepatitis B core antigen. We observed that measles virus needs a higher enzyme concentration than yellow fever, adenovirus, dengue and respiratory disease viruses. Lung and pancreas tissues infected by adenovirus were treated by Chandler & Gorelkin (1983) with 0.25% trypsin for 1 hour at room temperature with similar results as obtained by us using 0.1% trypsin for 2 hours at 37°C. The partial substitution of trypsin by pepsin (Reid et al., 1983) did not give better results in our experience.

In a previous paper (Schatzmayr et al., 1984) we examined by direct immunofluorescence liver sections from 76 patients (Figs. 9 and 10) of yellow fever, with 34 positive results.

However, attempts to detect virus particles by transmission electron microscopy in liver fragments from three of them with positive diagnosis by immunofluorescence, were unsuccessful.

The trypsin-immunofluorescence technique in our hands is a useful method for both recent and retrospective diagnosis or when histopathology is doubtfull.

The use of direct or indirect immunofluorescence depends upon the available conjugates. The fluorescence intensity depends both from antigens exposed and from the conjugate which must be tested for each sample.

Conjugates prepared in our laboratory against dengue type 1 and yellow fever could be used in a direct fluorescence method with reproducible results, reducing the number of reagents and steps in the procedure.

The trypsin-immunofluorescence technique described can be well adapted to routine histopathology, since neither the formalin fixative nor the standard paraffin embedding medium have to be changed.

RESUMO

Detecção de infecção viral por imunofluorescência em tecidos fixados em formol, pré-tratados com tripsina — A presença de antígeno viral em cortes de tecidos humanos fixados em formol e emblocados em parafina foi demonstrada pela digestão com tripsina seguida de imunofluorescência direta ou indireta. Os espécimens podem ser utilizados para diagnoses retrospectivas. A técnica da imunofluorescência deve ser adaptada à infecção viral suspeita segundo diagnose histopatológica prévia. Os parâmetros para a digestão do tecido pela tripsina, relacionados à concentração, duração de atuação e temperatura, expõem diferentes antígenos virais e devem ser previamente testados para cada sistema a ser estabelecido. Uma digestão mais intensa deve ser aplicada para a detecção do vírus do sarampo em tecido pulmonar do que para adenovírus ou vírus respiratório sincicial no mesmo tecido. Por outro lado, o vírus da febre amarela em tecido de fígado necessita de uma digestão mais fraca.

Palavras-chave: diagnose viral — imunofluorescência — diagnose retrospectiva — digestão por tripsina
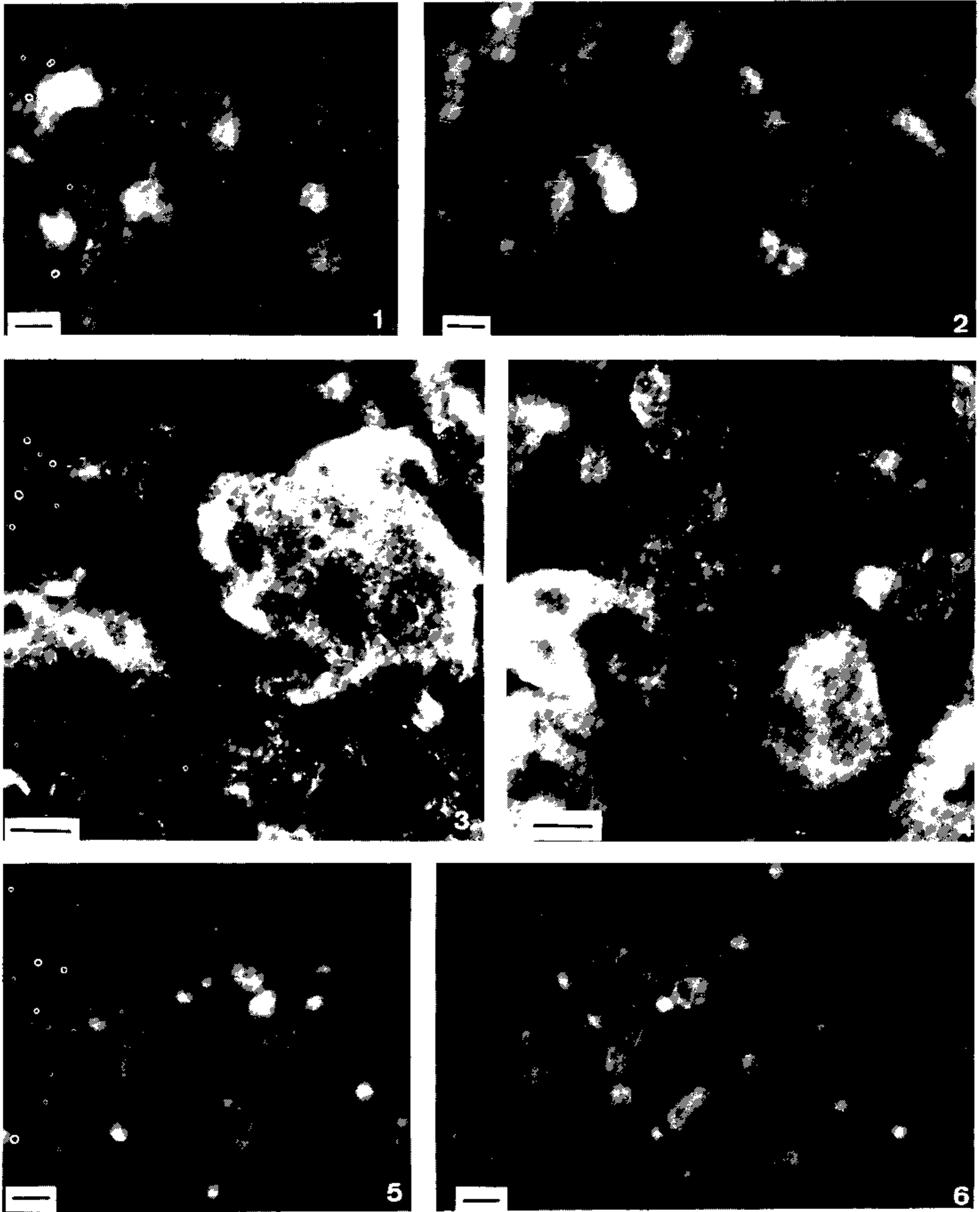
Figs. 1-2: Immunofluorescence of adenovirus infected pulmonar tissues (500x). Figs. 3-4: Immunofluorescence of giant cells in pulmonar tissues infected by measles virus (800x). Figs. 5-6: Weak immunofluorescence of pulmonar tissue infected by respiratory syncytial virus (500x). Bar = 10μ.
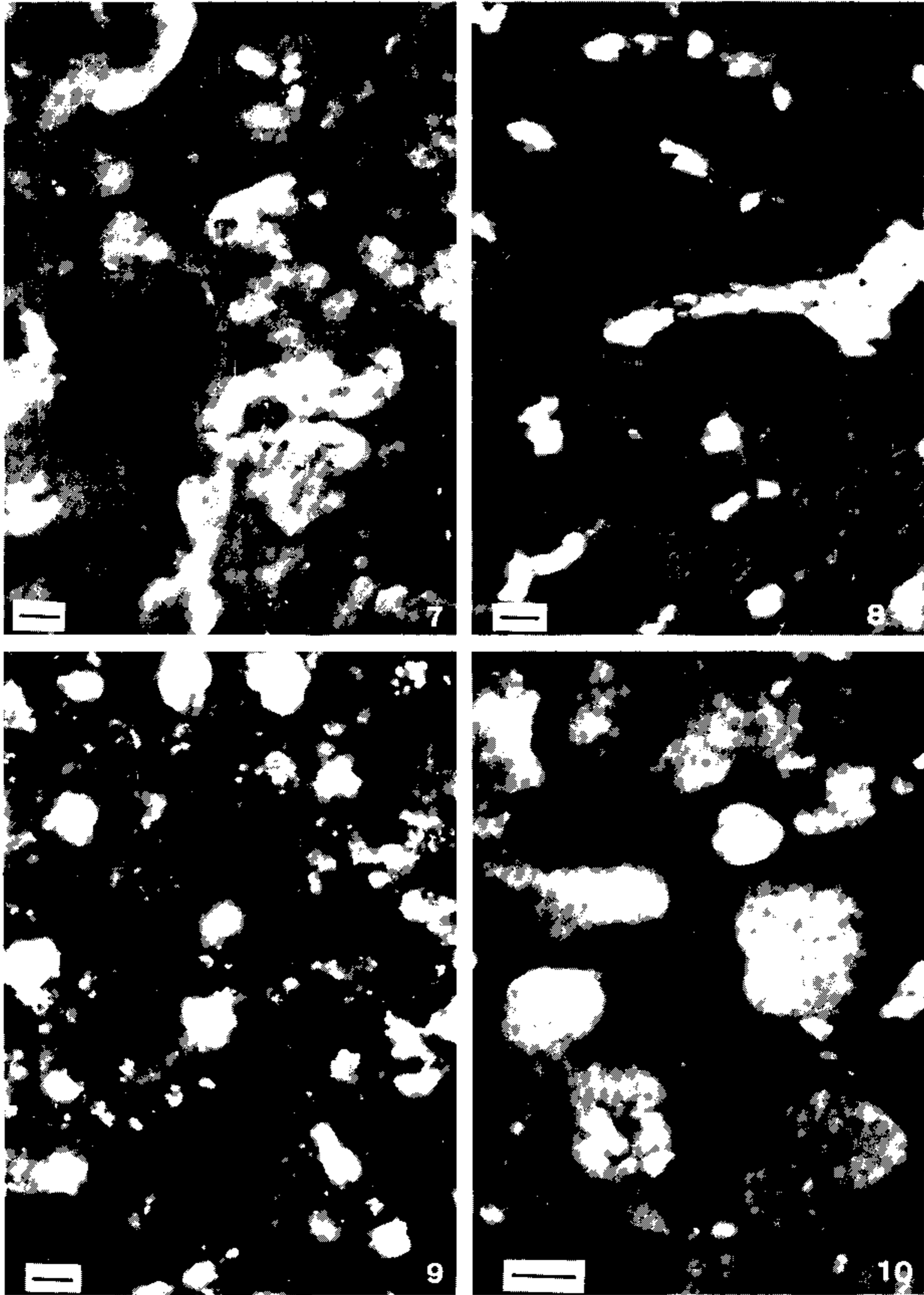
Fig. 7: Immunofluorescence of pulmonar tissue infected by parainfluenza virus (500x). Fig. 8: Negative immunofluorescence of liver tissue tested for yellow fiver infection. Lymphocytes present a strong autofluorescence (500x). Figs. 9-10: Immunofluorescence of liver tissue infected by yellow fever virus (500 and 800x). Bar = 10μ.

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