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LABORATORY DIAGNOSIS OF RABIES BY THE FLUORESCENT ANTIBODY TEST APPLIED TO BRAIN TISSUES OF EXPERIMENTALLY INOCULATED MICE AND EITHER PRESERVED IN FORMALIN OR UNDER REFRIGERATION

DIAGNÓSTICO LABORATORIAL DA RAIVA PELA REAÇÃO DE IMUNOFLUORESCÊNCIA DIRETA APLICADA A TECIDOS CEREBRAIS CONSERVADOS EM FORMOL OU EM REFRIGERAÇÃO, OBTIDOS DE CAMUNDONGOS EXPERIMENTALMENTE INFECTADOS

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SUMMARY

The fitness of formalin - treated materials for use in rapid rabies diagnosis was evaluated through Fluorescent Antibody (FA) test, using the digestion technique of pepsin and trypsin and the impression method of slide preparation. To achieve this proposal, brain fragments of experimentally rabid mice were submitted to different treatments of brain preservation by either using. pH adjusted formalin solutions or refrigeration, and the FA tests were run at ten experimental phases for a test period of 28 days. The results of FA reactivity ranged from 90.0% to 58.0%, depending on the treatments submitted; under the condition of the experiment, brain specimens should be maintained at refrigerating temperature until 96 hours for suitable FA examination, over this period the results should be irregular due to tissue degradation. The preservation of brain fragments in formalin, with further enzymatic digestion of pepsin and trypsin and the use of impression method did not mask or alter the virus antigenicity for adequate identification through FA technique, although the procedures should never substitute the existing methods of rapid rabies diagnosis.

UNITERMS: Rabies, diagnosis; Fluorescent Antibody Technique; Trypsin; Pepsin

INTRODUCTION

Since the early description of the fluorescent antibody (FA) staining in rabies by GOLDWASSER; KISSLING[7] (1958); the suitability of this technique in revealing rabies antigens in tissues which have been formalin-fixed and paraffin-embedded has been demonstrated by several authors[3, 6, 10, 11, 12, 13].

HUANG et al.[9] (1976) introduced the trypsin digestion of the paraffin-embedded sections to ameliorate the immunofluorescent staining; CURRAN; GREGORY[5] (1977) reported that the duration of enzymatic treatment is of great importance for restoration of immuno-histochemical reactivity of formalin-treated and paraffin-embedded sections. BROZMAN[1] (1978) investigated the action of chymotrypsin on formalin-treated materials for the antigenicity restoration through immuno-histochemical analysis; formaldehyde did not induce protein denaturation and preserved the antigenic determinants without loosing the binding capacities to antibodies (BROZMAN[2], 1980).

KENNETH et al.[10] (1980) have demonstrated the presence of rabies antigens in trypsin - treated human and mice histologic sections by means of immunofluorescence; HED; ENESTRON[8] (1981) reported the masking effect of antigenicity of formalin in the presence of proteins and that pepsin digestion does not change the immuno-histochemical reactivity of the antigens. UMOH; BLENDEN[15] (1981) evaluated the FA staining of rabies virus antigens in smears of formalin-treated and trypsin-digested tissues; the advantage of using smears resides on the elimination of the time-consuming procedures needed for paraffin sections.

The effect of trypsin digestion alone or pepsin-trypsin digestion of formalin-fixed brain tissues with an increased immunofluorescent staining of pepsin-trypsin double step treatment was reported by REID et al.[12] (1983); PALMER et al.[11] (1985) also found similar results with the double step digestion technique for demonstration of rabies virus antigens through FA and unlabeled antibody enzyme method.

Preservation of tissues in formalin, however, is not a procedure recommended for rabies diagnosis because it precludes the animal inoculation test[14]; refrigeration or freezing is more practical nowadays. But still it is not uncommon to laboratories receive materials preserved in formalin with request of rabies diagnosis.

For this purpose, histologic examination of paraffin-embedded and enzyme-digested sections through FA method is a time-consuming procedure not fitted for a rapid routine rabies diagnosis.

The use of impression method of slide preparation, instead of histologic section would save time for the establishment of the diagnosis when processing materials that have been treated already by formalin.

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This work is aimed to evaluate the fitness of the formalin-preserved and enzyme-digested tissues for the direct FA examination using the impression method of slide preparation.

To achieve this purpose, brains of rabid mice were either preserved in formalin or kept under refrigeration without any preservatives and were sequentially evaluated through FA test for inclusion bodies for a test period of 28 days.

MATERIAL AND METHOD

A fresh canine street rabies virus isolate M-17/89* was used for mice inoculation. Following isolation, the virus had been stored at - 20°C, thawed and been maintained in mice through three additional intracerebral inoculation and the titer determined at the time of inoculation was $10^{3.8}$ LD_{50}/0.03 ml in CH-3 Rockfeller albino mice. The isolate had been previously identified by means of serum neutralization test, using rabies hyperimmune equine** serum as a positive control serum.

The diluent used contained normal foal serum without antibodies against rabies, at a concentration of 2% (V/V) in distilled water with 1.000 IU of penicillin and 1.25 mg of Streptomycin per ml.

The FA procedure was accordingly to the method of GOLDWASSER; KISSLING[7] (1958) with slight modification[4]; the fluorescein-labeled antirabies conjugate*** was used at a working dilution of 1:60.

Pepsin**** was used at a concentration of 2 grams in 500 ml 0.01M PBS; trypsin***** at a concentration of 1.25 gram in 500 ml 0.01M PBS, according to REID et al.[12] (1983), and KENNETH et al.[10] (1980).

The formaldehyde solution was prepared by making a 10% solution of a commercial formalin****** in 0.01M PBS; two stock solutions of 500 ml each were prepared; the first stock solution had adjusted the pH to 7.6 with 0.1N NaOH, the second one was adjusted to pH 7.5 by adding 100 grams of a commercial chalk*******.

Experimental procedure - Sixty-five mice were inoculated 0.03 ml of virus suspension containing approximately $10^{3.0}$ LD_{50} through intracerebral route; at 11th post inoculation day 50 agonizing mice were killed and randomly separated into 10 groups of 5 mice each, tagged with individual coded number and brains were then collected. Each brain had been pressed through its basal portion against the microscope slides and each brain slides were made in fourfold, air dried and fixed in acetone (-20°C) for at least 4 hours and then stored at -20°C until further FA processing. Impressions prepared in this way were identified as "treatment D". Subsequently, each brain was cut into three portions of approximately equal size and fragments were submitted to one of the following treatments:

- Treatment A - Brain fragments treated with 10% formalin solution in 0.01M PBS with NaOH adjusted pH;

- Treatment B - Brain fragments treated with 10% formalin solution in 0.01 PBS with chalk adjusted pH;

- Treatment C - Brain fragments stored in refrigerator (4° - 9°C) without any preservatives.

Materials were processed in 10 experimental phases; for each phase a group of five formalin-treated brain fragments were digested daily until the 7th day; afterwards were made at weekly interval, i.e., at 14th, 21st and 28th day of preservation.

For enzymatic digestion, the method used was of the double step digestion; the first step was made by using pepsin, according to the work of REID et al.[12] (1983), the second step, with trypsin, as described by KENNETH et al.[10] (1980). The formalin-treated fragments were cut into pieces of approximately 3 x 3 mm, rinsed with water and washed for 10 minutes with 0.01M PBS; digested with 0.4% pepsin, pH 2.5 at 37°C for 60 minutes; washing with 0.01M PBS for 10 minutes and further digestion with 0.25% trypsin, pH 7.6, at 37°C for 90 minutes. After digesting, fragments were washed in 0.01M PBS for 10 minutes, placed on a wooden tongue-depressor with cut surface facing upward, air dried at room temperature for additional 10 minutes and impressions were made by pressing the fragments against glass slides. Impressions of refrigerated fragments were made similarly and, independent to the treatments submitted, all slides were fixed in acetone at -20°C, air dried and then kept at -20°C until the next day for FA staining.

For FA examination, the negative result was accepted if all fourfold slides were negative; a positive result was assumed if at least one slide were found positive, according to the following criteria:

Negative (-) = absence of intracytoplasmic inclusion bodies and/or "sandlike" particles;

Positive (+) = presence of intracytoplasmic inclusion bodies and/or "sandlike" particles.

RESULTS

The results of the FA examinations made on brains of rabid mice that had been submitted to different treatments of preservation are summarized in Tab. 1.

The immunofluorescent reactivity of rabies antigens was seen in 100.0% of the examined control slides prepared from fresh

* From "Centro de Controle de Zoonoses: Secretaria de Higiene e Saúde of the São Paulo City.
** Kindly provided by the "Institute Butantan", São Paulo.
*** From the "Instituto Biológico de São Paulo" - SP.
**** Pepsin - Difco.
***** Trypsin - Merck Sharp & Dhome.
****** Indústria Comércio Dubon Ltda.
******* Grupo Química Industrial Ltda.

TABLE 1
Results of fluorescent antibody examination of brain impressions of experimentally rabid mice, according to treatments and time of conservation. São Paulo, 1990.

Experimental Phase	Condition Time of conservation (days)	Treatment* A	B	C	D
1		4/5	4/5	5/5	5/5**
2	2	4/5	5/5	5/5	5/5
3	3	4/4	5/5	5/5	5/5
4	4	5/5	4/5	3/5	5/5
5	5	5/5	3/5	1/5	5/5
6	6	4/5	4/5	2/5	5/5
7	7	5/5	5/5	4/5	5/5
8	14	5/5	5/5	4/5	5/5
9	21	5/5	5/5	2/5	5/5
10	28	4/5	4/5	4/5	5/5
Total	...	45/50	44/50	29/50	50/50
Sensitivity		90.0%	88.0%	58.0%	100.0%

* Treatment: A - formalin + PBS stabilized with 0.1N NaOH;
B - formalin + PBS stabilized with chalk;
C - refrigeration;
D - fresh/frozen.
** nº positive slides/total slides examined.

brains (treatment D). Regarding treatments A, B and C, the first two corresponded to formalin-treated groups and the third one, only refrigerated, were found respectively with 90.0; 88.0 and 58.0% of positive reactivity. These values were derived from an overall analysis of the entire test period of the 28 days.

Each diagnostic run made at different experimental phases was numbered from 01 to 10, the proportion of positive results has varied during this period; greater variation was found with the results of treatment C, at 5th day of conservation only one brain was found positive out of the five examined.

Statistical analysis through Cochran's non-parametric method and binomial test indicated a lack of significant difference at level of 0.05 when results of treatment A versus treatment B were compared each other.

DISCUSSION

The sensitivity of the FA test applied to formalin-treated and enzyme-digested brains was found with slight variation. These values between treatment A (90.0%) and treatment B (88.0%) were found without any statistically significant difference at α=0.05; thus chalk can alternatively be used in place of NaOH to correct the pH of formalin solution. The necessity to stabilize the formalin solution was discussed by REID et al.[12] (1983); UMOH; BLENDEN[15] (1981) used a formalin solution with neutral pH, but procedure for its neutralization was not mentioned in their work.

The double step digestion with pepsin-trypsin described by REID et al.[12] (1983) was effective, the ideal digestion time in this experiment was 60 minutes for pepsin and 90 minutes for trypsin; under or above these times the results were irregular. BROZMAN[2] (1980) stated that the enzymatic digestion exposes the antigenic sites, letting the antibody-antigen interaction and facilitating the restoration of formalin-treated materials for examination under immuno-histochemical techniques.

Emphasis must be given for the working dilution of the FA conjugate used for enzyme - digested tissues, the conjugate hadd to be 3 or 4 times more concentrated than the usual dilution used for examination of fresh brains; this fact was reported previously by REID et al.[12] (1983).

In this experiment the pH of the formalin solutions was adjusted to 7.5 and 7.6, in order to decrease the acidity of formalin solution; the acidity could lower the working dilution of the conjugate, since acidic pH may interfere on the FA staining by quenching of fluorescein at low pH values. With specimens stored in refrigerator, the reduction of positive results was found around the 4th to 5 th day of conservation; this coincided to the tissue degradation that had exacerbated at about the same time, making the impressions on the slides extremely difficult to be obtained. These slides were also found with lots of "sandlike" particles and even with conjugate precipitates, making the interpretation difficult according to the criteria used.

Difficulty was also found in making impressions from enzyme-digested fragments due to the poor adherence of the tissues, thus fourfold examination of the same material proved to be valid.

The refrigerated tissues, in our point of view should be examined until the 4th day of conservation; over this period it may lead to irregular false positive or false negative results difficult to be explained, as occurred in this experiment at the last phase.

The formalin has preserved adequately the antigenicity of rabies virus antigens even after nearly a month of conservation and a masking effect was not found in this experiment. After enzymatic digestion, the reactivity of rabies antigens to FA test could be restored promptly.

The procedures described in this paper should never substitute the routine technique applied for rabies diagnosis because of some pitfalls. It should be employed, however, as an auxiliary diagnostic tool for clarifying rabies diagnosis in an unusual situation when tissues have been fixed already in formalin.

RESUMO

Avaliou-se a adequação do emprego de cérebros preservados em formol para o estabelecimento rápido do diagnóstico da raiva pela reação de imunofluorescência direta, utilizando a técnica de digestão enzimática de pepsina e tripsina e método de impressão para o preparo de lâminas. O delineamento proposto contou com fragmentos de cérebros de camundongos experimentalmente infectados submetidos a diferentes tratamentos de conservação, com o uso de soluções de formol com pH corrigidos, ou submetidos à refrigeração; os testes de imunofluorescência foram realizados em 10 fases experimentais, por um período de 28 dias. Os resultados da prova de imunofluorescência variaram de 58.0% a 90.0% de positividade, dependendo dos tratamentos dispensados. Nas condições do experimento, os materiais destinados à prova de imunofluorescência podem ser conservados em temperatura

de refrigeração por até 96 horas; após este período aumentam os resultados irregulares devido à degradação tissular. Nos tecidos mantidos em formol e após digestão enzimática, com a aplicação do método de impressão, observou-se o fenômeno de restauração da antigenicidade do vírus rábico, permitindo uma adequada identificação através da prova de imunofluorescência; no entanto, estes procedimentos não devem substituir os métodos atualmente empregados para o diagnóstico rápido da raiva.

UNITERMOS: Raiva, diagnóstico; Imunofluorescência; Tripsina; Pepsina

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