ANTIBODIES TO RAT BRAIN CHOLINE ACETYLTRANSFERASE: SPECIES AND ORGAN SPECIFICITY

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1. Introduction

The production of antibodies against enzymes involved in neurotransmission has been developed in the last few years. In the case of catecholamines, the use of antibodies against enzymes involved in their synthesis has led to the fine localization of catecholaminergic neurons by using immunohistochemical techniques [1].

Among the cholinergic enzymes, the presence of acetylcholinesterase (Ach E), a post synaptic enzyme, was similarly visualized [2]. However, Ach E is known to have a wider distribution than choline acetyltransferase (ChAc) which is located on the presynaptic side of the cholinergic synapse. It is thus clear that obtaining antibodies against ChAc might lead to a precise mapping of cholinergic pathways. Moreover, the use of a specific antibody could aid in understanding some of the structural features of the enzyme forms.

In order to obtain antibodies, we injected several purified preparations of rat brain ChAc into rabbits and other animals. In a previous paper [3] we described the antisera: no precipitating activity against ChAc was detected, but all antisera reacted in immunodiffusion with several proteins which contaminated the antigen.

Here, we report on the production of specific antibodies against ChAc as demonstrated by immunoprecipitation. These antibodies appeared after one year of continuous immunization of one rabbit. We also report on the species and organ specificity of ChAc, by using various immunological techniques, as well as on its immunological reactivity after heat inactivation.

2. Materials and methods

2.1. *Assays*

ChAc assay is based on Fonnum's technique: [^{14}C]acetylcholine derived from [^{14}C]acetyl-CoA is extracted as tetraphenylboron salt, in ethylbutylketone [4]. It was modified as previously described [3].

2.2. *Carnitine acetyltransferase assay*

This was done in a similar medium as the ChAc assay, but 10 mM carnitine (generous gift of Otsuka Pharmaceutical Ltd. Japan) was used instead of choline and no eserine was added. The reaction was stopped by the addition of 20 μl in 1 N formic acid. Following the acidification, acetyl-[1-^{14}C]carnitine was extracted by ethylbutylketone containing 10 mg of tetraphenylboron per ml and transferred to the scintillation vial. Omission of the acidification step gave the blank values.

Proteins are assayed following Lowry [5].

2.2. *Antigen purification*

Antigen was prepared as previously described [3]: i) Rat brains (630 gm wet weight) were homogenized in a buffer solution adjusted to pH 5; ii) After centrifugation at 10 000 *g*, the supernatant was discarded. The pellet was rehomogenized in 200 mM NaCl. After centrifugation, most of the activity was found in the

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Table 1

Step	Total units (μmole) Acetylcholine formed per min	Recovery (%)	Protein (mg)	Specific activity (μmoles/min/mg)
i Homogenate	40.5	100%	66 000	0.0007
ii Supernatant 200 mM NaCl	30.5	75%	3 960	0.0077
iii Sephadex CM eluate	25.8	64%	257	0.105
iv $(NH_4)_2SO_4$ precipitate	19.6	48%	46.4	0.422

supernatant; iii) It was diluted up to 100 mM NaCl and adjusted to pH 5.9. The enzyme was absorbed on Sephadex CM and eluted by a linear gradient. Fractions with a specific activity over 75 nmoles/min/mg of protein were pooled; iv) The enzyme was precipitated between 38.5% and 50% saturation by a saturated $(NH_4)_2SO_4$ solution at pH 6.

2.3. *Immunization schedule*

Rabbit no. 27 had been injected with 5 mg of ChAc (iv – $(NH_4)_2SO_4$) in a 1:1 complete Freund's adjuvant emulsion and boosted twice every two weeks. It was boosted again six months and twelve months later. A week after the last boost, the same amount of antigen without Freund's adjuvant was injected intradermally and on the following day intravenously. Bleeding obtained at that point will be named as 27 B. First bleedings obtained before the 6 and 12 months boosts will be named as 27 A.

2.2.5. Double immunodiffusion tests

These were performed following Ouchterlony's technique, in 1.2% agar gel [7].

2.2.6. Immunoprecipitation tests

The tests were made according to Yanofsky [7]

3. Results

3.1. *Obtention of bleeding 27 B and its analysis*

Table 1 summarizes the purification steps of rat brain ChAc, as described in Materials and methods. The specific activity obtained at the step (iv) represents a 600-fold purification. The procedure was found suitable for immunization, as ChAc was prepared within three days, so that we injected, among various animals, ten rabbits with such a preparation of ChAc.

The story of Ra # 27 was rather unique: the first few bleedings obtained during the semester following the start of immunization have already been described [3] and will be quoted from now on as bleedings 27 A. The latest bleedings – bleedings 27 B – obtained after the rabbit had been boosted again twice (see Materials and methods), still gave in agar gels multiple bands of precipitation with the ChAc preparation, similar to that shown previously in the analysis of bleedings 27 A (see fig. 1 in ref. [5]).

However, whereas bleedings 27 A were devoid of any anti-ChAc activity in immunoprecipitation tests, it appeared that bleedings 27 B possessed, in contrast, specific antibodies to the enzyme (fig. 1). When a constant amount of ChAc was added to increasing concentrations of antiserum, immunoprecipitation occurred and residual activity was assayed in the supernatant. The 50% precipitation point corresponded to a 1:74 dilution of bleeding 27 B.

Antiserum 27 B seems specific to ChAc, as another acetyltransferase enzyme, such as carnitine acetyltransferase, is not immunoprecipitated by the highest antiserum concentration.

In order to visualize the difference in immunodiffusion between the inactive bleeding A and the active bleeding B, we absorbed the ChAc preparation with bleeding A. We checked that absorption was completed as shown by no further reactivity of bleeding A with it. In contrast, the absorbed ChAc preparation

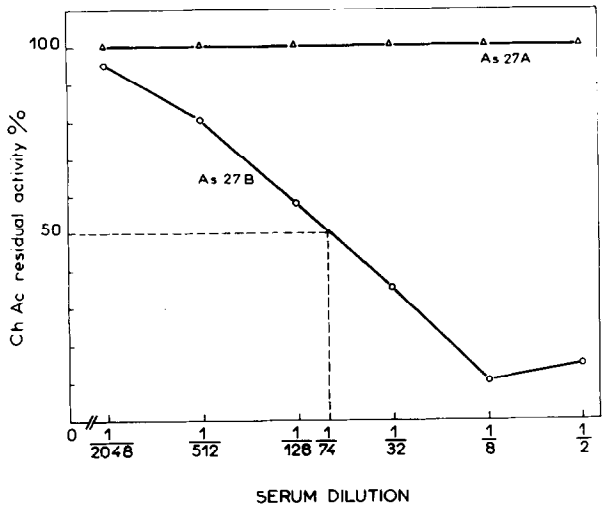

Fig. 1. Immunoprecipitation of rat brain ChAc by 27 A and 27 B. To a constant 250 pmoles amount of enzyme, serial dilutions of antisera 27 A and 27 B were added. The final volume, 50 µl, was incubated for 30 min at 37°C and for 48 hr at 4°C. After centrifugation at 2000 *g* for 10 min, the supernatant was assayed for residual ChAc activity. Control: preimmunization serum. Results: % of residual activity below control values. Antigen: 105 000 *g* supernatant of a rat brain homogenate in 0.15 M NaCl, Na phosphate buffer 10 mM, pH 7.2 (PBS), diluted with the same buffer to obtain the appropriate concentration of ChAc.

still reacted with bleeding 27 B in giving a single precipitation band (fig. 2). We then postulated that this band was likely to correspond to antibodies responsible for precipitation of ChAc in the Yanofsky type immunoprecipitation tests.

3.2. *Immunological reactivity of ChAc*

3.2.1. Heat treatment

We studied the effect of heat inactivation on the immunological reactivity of our ChAc preparation. ChAc is very sensitive to heat inactivation, as heating for 10 min at 47°C is followed by a complete loss of enzymatic activity. However in agar gels, the heat inactivated enzyme gave a precipitation line having a reaction of identity with the native preparation.

In immuno precipitation, after heating at 54°C for 30 min, increasing amounts of treated enzyme inhibited the precipitating action of antiserum 27 B on a constant amount of native enzyme (fig. 3). The inhibition was similar to that due to increasing amounts of untreated enzyme added to the antigen–antibody mixture, thus demonstrating immunological similarities between active and heat inactivated ChAc.

3.2.2. Species specificity

ChAc extracts of various brain vertebrates were compared in immunoprecipitation tests (fig. 4). Reac-

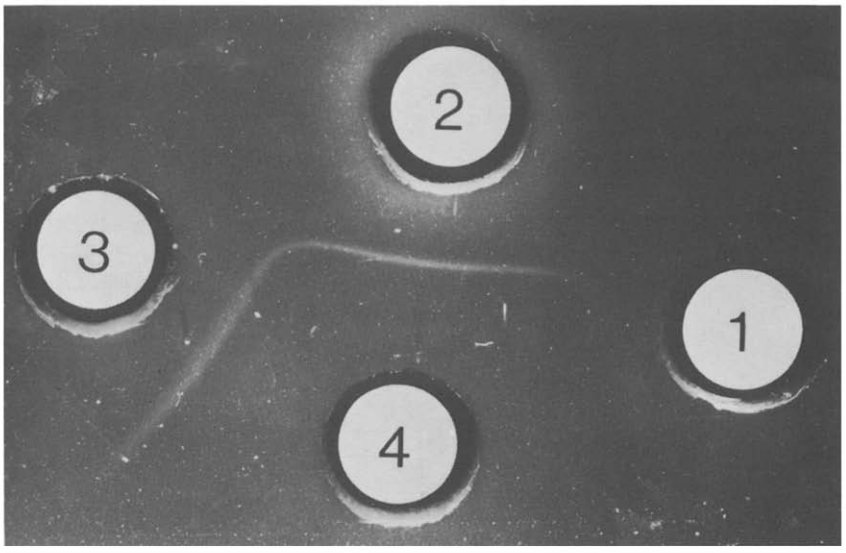

Fig. 2. Double immunodiffusion of antiserum 27 A and 27 B against immunoabsorbed ChAc: Well 1, antiserum 27 A; Well 2, antiserum 27 B; Well 3, antiserum 27 B diluted 1:2; Well 4, ChAc step $(NH_4)_2SO_4$, 3.6 mg/ml of protein immunoabsorbed by antiserum 27 A.

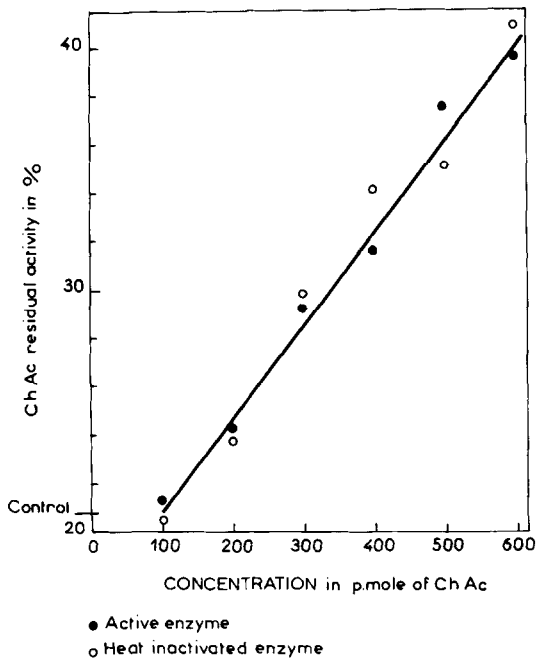

Fig. 3. Inhibition of 27 B by heat inactivated enzyme Antigen: 105 000 *g* supernatant of rat brain homogenate in PBS. A part of it was heat inactivated at 54°C for 30 min. To a constant 100 pmoles amount of enzyme and to a constant 2.5 μl amount of 27 B, variable amounts of inhibitor were added. The final reaction mixture was adjusted to 40 μl with PBS. The inhibitor was either active enzyme or heat inactivated enzyme. After 30 min at 37°C and 48 hr at 4°C, the reaction mixture was centrifuged and supernatant assayed for ChAc. Each point represents % of residual activity. Similar reaction mixture with preimmune serum instead of 27 B was used as reference.

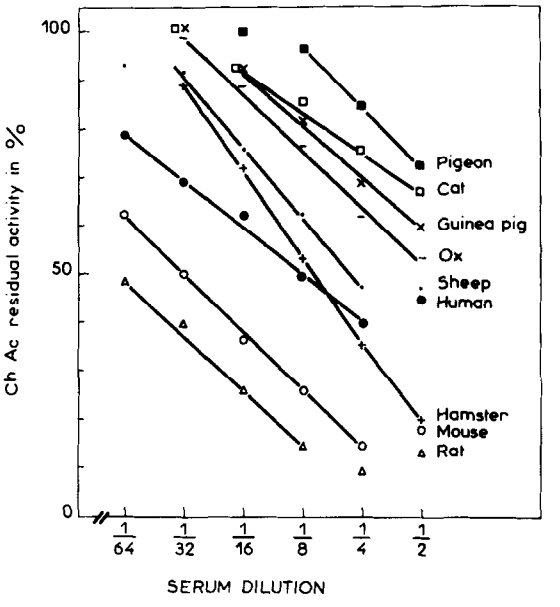

Fig. 4. Immunoprecipitation of ChAc from different species by 27 B. Methods and results expression as in fig. 1. Antigens: 105 000 *g* supernatant of brain homogenate in PBS. Whole brain from rat, mouse, guinea pig, hamster and pigeon was homogenized directly after decapitation of the animal. Cat striatum was dissected on anaesthetized animal. Ox and sheep striatum were dissected 1 hr after slaughtering. Human frontal cortex, from a 63 year-old man, removed at the time of a surgical procedure.

tivity of antiserum 27 B with all the mammalian brain extracts was observed. This was also the case with bird brain. However no reactivity was seen with frog brain extracts and *Torpedo* electric organ extracts.

The concentration of serum corresponding to the 50% precipitation point of ChAc was relatively close when rat and mouse enzymes were compared (table 2). The relative immunological distance for other species are expressed by the factor by which serum concentration must be raised in order that a particular ChAc extract gave 50% precipitation. A correlation seems to exist between the cross reactivity and the isoelectric point measured in the different species. Cat brain excepted, the more acidic the isoelectric point, the weaker the cross reactivity.

3.2.3. Organ specificity

The immunoprecipitation pattern was found to be similar in all rat nervous tissue extracts tested, i.e. brain, sympathetic ganglia and neuromuscular junction.

Because of immunological similarities of rat- and mouse-brain enzyme, we tested the mouse neuroblastoma clone NS 20 (a gift from M. Nirenberg), a cell line known to be very rich in ChAc [9]. We found the cell line to have a reactivity similar to that of mouse brain, giving an opportunity of testing ChAc arising from a distinct type of nervous cell.

Moreover, in the search of a differentiation pattern along development, we tested new-born rat brain extracts, which gave an immunoprecipitation pattern similar to that of adult rat brain extracts.

Finally, we had an opportunity to compare two human ChAc extracts, one from brain, the other from placenta (fig. 5) which is the only non-innervated or-

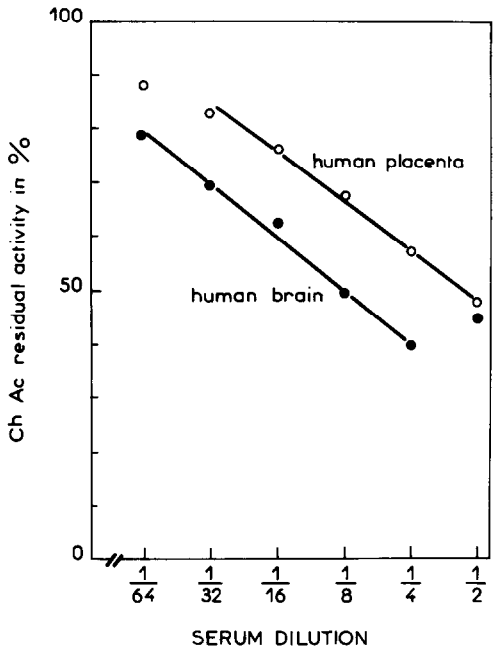

Fig. 5. Immunoprecipitation of human brain and human placenta ChAc by 27 B. Methods and results expressed as in fig. 1. Brain antigen: supernatant of homogenate in PBS of frontal cortex. Placenta similarly treated was obtained at delivery.

Table 2
Indices of immunological distances for ChAc in brain extract of various species. Correlation with isoelectric points.

Species	Serum dilution corresponding to the 50% precipitation point	Reactivity indices	Isoelectric points
Rat	$\frac{1}{74}$	1	7.4, 7.8, 8.4
Mouse	$\frac{1}{37}$	2	7.1, 7.4, 8.4
Human	$\frac{1}{11}$	7	
Hamster	$\frac{1}{8}$	9	
Sheep	$\frac{1}{6}$	13	
Ox	$\frac{1}{3}$	25	
Guinea-pig	$\frac{1}{2}$	38	6.8
Pigeon	(1.3)	98	6.6
Cat	(1.5)	107	7(7.6), (8.41)

Serum dilution: Values corresponding to the 50% precipitation point and calculated from data shown in fig. 3. For pigeon and cat, they are extrapolated.
Reactivity indices: Factor by which antiserum concentration must be raised in order that a particular ChAc extract gives a 50% precipitation value equal to that of rat (rat index: 1).
Isoelectric points: data from Malthe Sorenssen and Fonnum [8].

gan rich in ChAc. Reactivity indices expressed in the same manner as that described in table 2, were respectively 7 and 28 for brain and placenta enzyme, which demonstrates a rather large immunological distance between the two forms.

4. Discussion

It was perhaps a chance observation that, among so many animals of various origins injected with our ChAc preparation a single rabbit revealed itself as producing an active anti-ChAc antiserum, a year after the start of its immunization. Anyway we do not know if the difficulty in obtaining specific antibodies to ChAc is related to a tolerance phenomenon, which at least in primates might be due to exposure of fetuses to placental tissues rich in ChAc. Furthermore we demonstrate here the wide cross-reactivity of our antiserum with ChAc from various species. One might thus assume that ChAc underwent little change during evolution.

Having in mind a biological application, i.e. an immunohistochemical use, of such an active antiserum we looked for further purification of bleeding 27 B, with the hope of getting monospecific antibodies to the enzyme. As bleeding 27 A and 27 B differed only by the presence of antibodies against ChAc, we combined the use of immunoabsorbents in various ways to obtain finally monospecific antibodies against ChAc (unpublished data).

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