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CRITERION OF CURE OF HUMAN CHAGAS' DISEASE AFTER SPECIFIC CHEMOTHERAPY: RECENT ADVANCES

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During the chronic phase of Chagas' infection diagnosis is in general achieved by conventional serology (CS) which detects specific immunoglobulins against *Trypanosoma cruzi*. The antigens for CS are total extracts, purified fractions or whole fixed epimastigotes which are obtained, rather easily, by cultivation of the parasite on liquid medium. Among different CS tests the most common are: indirect immunofluorescence (IIF); complement-fixation reaction (CFR) or Guerreiro-Machado reaction; direct agglutination of trypsin-treated epimastigotes; indirect hemagglutination (HA); immunoenzimatic and radioimmune tests (Camargo & Takeda, 1979). However, there are evidences that most treated individuals remain with the CS positive in spite of persistently negative hemocultures and/or xenodiagnosis (Cançado et al., 1979; Cerisola, 1977). Therefore, CS, important for diagnosis purposes, seems to have limits in the control of cure.

Several evidences accumulated after studies of treated patients, in a 5 years follow-up as discussed here and partially published (Krettli, Cançado & Brener, 1982, 1983, 1984a, b) in parallel with experimental data (Krettli & Brener, 1982; Krettli, 1982; 1983; *plus* our enclosed chapter) indicate that antibodies detected by CS exist in the total absence of infection. Such is the case of mice either immunized with parasite purified fractions or surely cured by chemotherapy and some treated patients. However, as we have shown, none of those groups have circulating immunoglobulins directed against epitopes expressed on the membrane of living trypomastigotes (ALBA = anti-living bloodstream or lytic antibodies) as detected by complement-mediated lysis (CoML). Various methods used to detect ALBA are described elsewhere (Krettli, enclosed paper) and evidences are provided that ALBA are the same protective antibodies responsible for the acquired resistance displayed by the infected hosts. Based on such data we have suggested that antibodies detected against living trypomastigotes, but not by CS only, are a reliable marker of ongoing infections.

An analysis of data from almost 300 chagasic patients, among treated or untreated is herein discussed and resulted on a temptative classification of the treated ones based on negativation of the CoML test repeatedly performed. Two patients studied during the course of treatment and which became ALBA negative have allowed to the conclusion that these functional antibodies may disappear after 5-7 months of an effective treatment but that CS persists for up to 4 years or more.

MATERIAL AND METHODS

Patients with Chagas'disease: a total of 116 untreated and 156 treated chagasic patients were used, mostly adults, male and female, clinically defined and treated by Prof. Cançado who has followed-up some of these cases for up to 18 years. Two compounds have been used in long-term treatment, a nitrofuran derivative [3-methyl-4(5'-nitrofurfurylidene-amino)-tetrahidro-4H-1, 4-thiazine-1,1-dioxide] (nifurtimox) and a nitroimidazol derivative [N-benzyl-2-nitro-1-imidazolacetemide] (benznidazol) in a daily dose of 7-8mg/kg weight, by oral route during about 40 days. Details of treatment, intolerance, clinical indications, allergic problems, etc. are discussed elsewhere (Cançado et al., 1979; Cançado, 1985; Brener, enclosed paper).

Non-chagasic group: represented by 93 individuals mostly from the clinics of Prof. Cançado, therefore belonging to a similar economical status and geographical region of the chagasics. Healthy blood donors from a Hospital Blood Bank in town were also included. All of them were considered non-chagasic on the basis of at least 3 different negative serologic reactions (IIF, CFR and HA).

Sera: collected and processed at Hospital Medical School, the sera were sent in 2-3ml aliquots on ice, codified. They were kept at $-20^{o}C$ until used. Decodification was only performed after the tests by at least two of the investigators. On average 15-20 diluted sera (2 and 4 fold) plus controls were easily assayed each time by one person.

CoML test: used for detection of protective antibodies, also referred as lytic antibodies, is discussed in details in our enclosed chapter. Briefly it consists of incubating 2-4-fold diluted test sera with living trypomastigotes isolated from blood or cell cultures and then with fresh human complement (C). Three controls for each test are always included i.e. normal serum, a positive serum from an untreated patient; a "dissociated" serum (CS positive, CoML negative) from a treated patient. The controls are incubated with

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trypomastigotes (Try) as above and then with C and with heat inactivated human serum. The percent of lysis is evaluated counting the motile Try on test and control tubes. The percent of lysis is calculated by the equation:

$$\% \text{ lysis} = 100 - \frac{\text{no. try with test sera plus C X 100}}{\text{average no. try with normal sera plus C and inactivated C}}$$

This modification on the original equation (Krettli, 1978; Krettli, Weisz-Carrington & Nussenzweig, 1979) minimizes the error resulting from the low counts in tubes with agglutinated Try observed with most ALBA positive sera and inactivated C.

IIF with living Try: this technique is also described in our enclosed chapter and it briefly consists of treating Try pre-incubated with diluted sera in the presence of sodium azide at 4°C then washed and fixed, *plus* a fluorescein-conjugated anti-human IgG.

Conventional serology (CS): two tests are routinelly performed with all sera arriving at the Serology Section, Centro de Pesquisas René Rachou, by Miss Rosa M. Brigido Nunes, responsible for the laboratory, i.e. the indirect immunofluorescence test with formaline fixed epimastigotes *plus* fluorescein conjugated anti-human total immunoglobulins and the complement-fixation reaction (Camargo & Takeda, 1979). Sera are tested blind and decodification was together with the tests of CoML.

RESULTS AND DISCUSSION

CoML in untreated patients: the positivity of this test performed in 116 untreated chagasic patients was very high (97%) and similar to the rate obtained by the IIF (96%) but higher than with the CFR (90%). A total of 191 CoML tests were performed in this group and only 6 of them (from 4 different individuals) were false-negative (Table I). However, the negative patients were not the same in the three tests. This level of sensitivity found in IIF and CFR tests is in accordance with the literature.

In the case of false-negative CoML the failure was attributed either to: (a) the anti-complementar activity of the sera which can be abolished by using concentrated serum immunoglobulins obtained after precipitation with a saturated solution of ammonium-sulphate [$(NH_4)_2SO_4$]; (b) possible shedding of the antigen-antibody complexes from the surface membrane of the parasite at 37°C, a problem now overcame by using a 4°C temperature for incubation as previously described (Krettli, Pereira & Brener, 1983) as well as the addition of sodium azide. These procedures have abolished two pitfalls of the test, i.e. the anti-complementar false-negative results and shedding phenomenon; and iten *b* has diminished the intense variability of the serum lytic activity (30 to 90% within the same serum). Furthermore, in two untreated patients with a false-negative CoML, ALBA antibodies were demonstrated by the IIF against living Try. Among the 93 control non-chagasic all the 108 CoML tests were negative (Table I).

TABLE I

Proportion of negative tests in three different serologic reactions (CoML = Complement mediated lysis; IIF = indirect immunofluorescence; CFR = complement-fixation reaction) performed with sera from chagasic patients treated or untreated and from non-chagasic individuals

Groups	No. patients	No. negative tests/total performed (%)		
		CoML	IIF	CFR
Chagasic untreated	116	6/191 (3%)	11/252 (4%)	30/254 (10%)
Non chagasic	93	108/108 (100%)	106/113 (94%)	108/114 (95%)
Chagasic treated (total = 158)				
A – Uncured	93	46/579 (8%)	24/757 (3%)	106/758 (14%)
B – "Cured"	10	51/61 (83%)	52/75 (71%)	51/61 (83%)
C – "Dissociated"	29	142/176 (80%)	78/273 (28%)	110/232 (47%)
D – "Oscilating"	24	97/292 (33%)	35/266 (12%)	92/292 (33%)

The titers of lytic activity of the positive sera are low ($\leqslant 1:16$) but the intensity of parasite lysis is rather high as shown in 10 untreated patients randomly selected from our files (Table II). Percent of lysis varied from 56 to 96 with a 2-fold diluted serum. In this case all tests were performed, as originally described, at 37°C. Interesting to note, pacient CFD who had borderline CS but high levels of lytic antibodies.

TABLE II

Results of conventional serology (CFR, IIF) and of complement mediated lysis test (CoML) using sera from 10 untreated chagasic patients

Name	CFR*	IIF Titer	% Lysis with sera diluted	
			1:2	1:4
AG	R	1:640	65	48
AFSP	R	1:640	56	49
APS	R	1:640	72	77
AFM	R	1:320	76	83
CFD	FR	1:80	88	82
ELM	R	1:320	71	66
FR	R	1:640	69	80
JAAG	R	1:320	81	82
JMR	R	1:640	96	94
LAM	R	1:640	73	80

CFR = Complement-fixation reaction
IIF = Indirect immunofluorescence with epimastigotes and anti-IgG conjugated
R = Reactive; FR = Borderline, weekly reactive.

CoML on treated chagasic patients: a total of 156 treated patients have been followed up from 2-5 years by means of CoML repeated either at every 3-4 months, or twice a year. A few of them, however, came only 2-3 times for serology. A temptative classification of these patients based on our results of CoML and CS is as follows (Table III).

TABLE III

Evolution of CoML and IFF tests in 156 chagasic patients submited to specific treatment

Groups*	CoML	IIF	Characterization	No. Patients	% Total treated Patients
A	+	+	Uncured	93	59.6
B	–	–	Cured	10	6.4
C	–	+	Dissociated (cured?)	29	18.6
D	+/–	+	Oscilating CoML (?)	24	15.4

* 116 chagasic untreated controls: 97% of positive CoML and 96% of positive IIF
93 normal controls: 100.0% of negative CoML and IIF.

Group A: of 93 individuals (59.6%) of the treated group in which both, CoML and IIF, remained unaltered as if the patients have not been treated. Therefore they are considered as a therapeutic failure. The total number of negative CoML (8%) was similar to that seen on the untreated groups (Table I) a result also observed for the CFR. At least part of this result may be attributed to false negative tests induced by anti-complementar serum and/or antibody shedding.

Group B: of 10 individuals (6.4%) of the treated group in which both, CoML and IIF have became mostly negative ($\geq$ 70% negative tests) therefore they are considered as cured. The total number of negative tests (Table I) was equal for both CoML and CFR (83%) and somewhat lower for the IIF (71%). The remaining positive tests were either sera of borderline titers (1:80 IIF) or of low activity (CoML and CFR) being inconsistly reactive. It is remarkable that among the 10 cured patients only 4 were totally negative in the CoML; 5 in the CFR and none in the IIF tests. Since one also observes in the normal non-chagasic group a limited rate of accuracy we may attribute the positivity of the tests to their limited specificity.

Group C: consisted of 29 treated individuals (18.6%) most of them extensively studied in the last 5 years or for at least 2 years. Their sera showed a consistent dissociation between CoML and IIF tests (Table I) only the latter being positive. Since the proportion of negative CoML (80%) was similar to Group B, we propose that Group C may also be "cured". However, we prefer to use a question mark ("cured"?) until we find a reliable parasitological method to surely exclude any *T. cruzi* infection in this group. The total number of serological tests and % negative is illustrated on Table I.

Because of the importance that such "dissociated" group may have, we lately concentrated our studies comparing it with individual of the "uncured" Group A, similarly treated but with CoML positive. Some of this date will be further discussed.

Group D: of 24 treated individuals (15.4%) with a consistent positive IIF but a fluctuating or oscilating CoML, i.e. with irregular results, sometimes positive or negative (Table III). The total number and % of negative results (Table I) show 33% negativation for CoML and CFR whereas for the IIF it was 12%. There are at least two different patient types on this group, i.e. (a) one whom will schwich to either the "dissociated" or eventually to the "cured" groups, respectively C or B; (b) another of patients with anticomplementar serum activity, causing the false-negative CoML and CFR, as discussed above for the untreated group. Indeed, some patients of Group D, do have ALBA positive as demonstrated by the IIF performed with living Try as well as with serum concentrated immunoglobulins (ammonium sulfate precipitation technique, as discussed on another enclosed chapter by Krettli).

Evolution of ALBA on treated patients of the "dissociated" group

A comparison between all the tests of CoML and conventional IIF in the treated groups discussed above and summarized on Fig. 1 shows that in the "dissociated" group the percentage of negative CoML was significantly higher than the IIF. The absence of ALBA on these negative sera was further confirmed by the negative IIF performed with living Try in several patients of the "dissociated" group. Most of our "dissociated" sera come from patients treated over 5 year ago, therefore in which CoML has been negative since the first assay (patient RNF). However, in a few patients (JFS, MHO) we were able to detect the switch from a positive to a negative CoML (Table IV). This occurred between 2-7 months after treatment whereas in the same group IIF remained positive for up to 7 years in the absence of CoML.

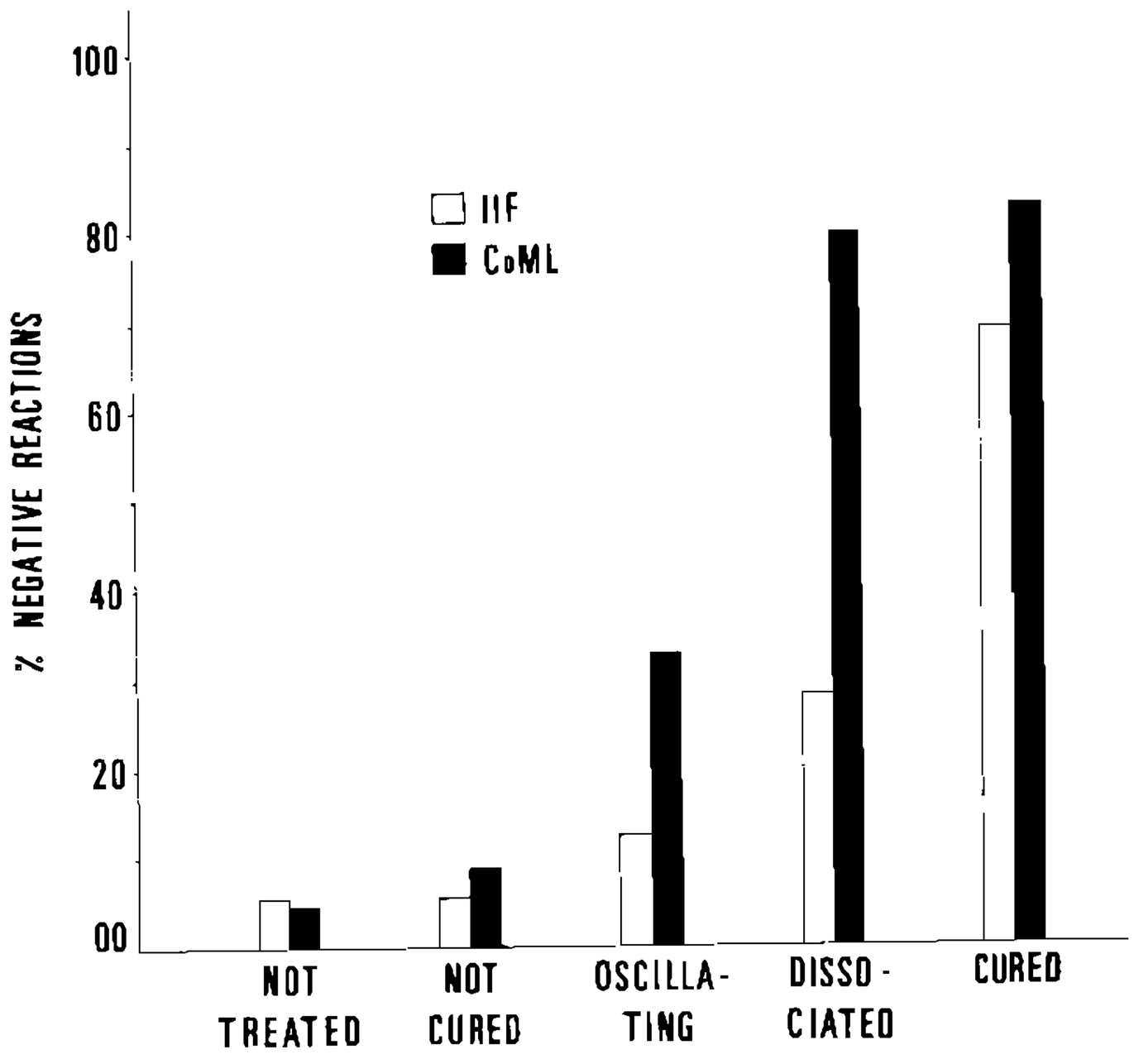

Fig. 1: total percentage of negative sorological reactions (CoML = complement-mediated lysis; IIF = conventional indirect immunofluorescence) performed with sera from various groups of chagasic patients, specifically treated and classified on Tables I and II.

As previously discussed we found a certain background of positive CoML in the "dissociated" group (17% of total tests positive – Table I, Fig. 1). Such positivity was, however, rather different from the CoML detected before treatment of the patient as well as in treated but uncured group A (Fig. 2). An intense lytic activity (50-100%) occurs with sera from patients IFR "uncured" and JFS and MHO, immediatly after treatment. Thereafter, the percent of lysis in the positive sera was bellow 30% in group C. Other patients of the "dissociated" group always gave negative CoML (ex. ACJ).

In spite of the persistent negative CoML the titers of conventional serology (IIF) were very high in group C as illustrated by patients JFS, MHO and RNF (Table IV). However, for reasons still not clarified, the IIF may also become gradually negative. An interesting patient who seems to be in this case (JFS) is illustrated on Fig. 3. Unfortunatelly he has not returned last year but we will not be surprised to find negative IIF at present on his serum. The conditions which had allowed 10 of the 156 treated patients (group B = "cured") to convert into negative by all serological reactions are not known. Must we wait 10 years more to see all our "dissociated" group to turn their CS also into negative?

TABLE IV

Antibody detected by conventional serology (indirect immunofluorescence = IIF) or by complement-mediated lysis (% CoML with diluted sera) in 3 patients at several months after treatment and from the group considered "cured"? (= dissociated)

Patient	Sera number	Months after treatment	Reciprocal of titers IIF	% CoML 1:2	% CoML 1:4
JFS*	3532	02	1:160	50	50
	3753	07	1:45	52	51
	3826	08	1:160	0	0
	4348	20	1:320	0	0
	4808	32	1:160	0	0
	5412	43	1:160	0	0
MHO	4533	01	1:640	67	62
	4705	02	1:640	53	41
	4834	05	1:640	0	0
	5297	15	1:640	0	0
	5567	21	1:640	0	0
RNF	5385	72	1:320	0	0
	6181	84	1:160	0	0
	6471	89	1:640	0	0

Figs. 2 and 3 have further data on patients JFS and MHO illustrating all sera tested during the 5 years of follow-up.

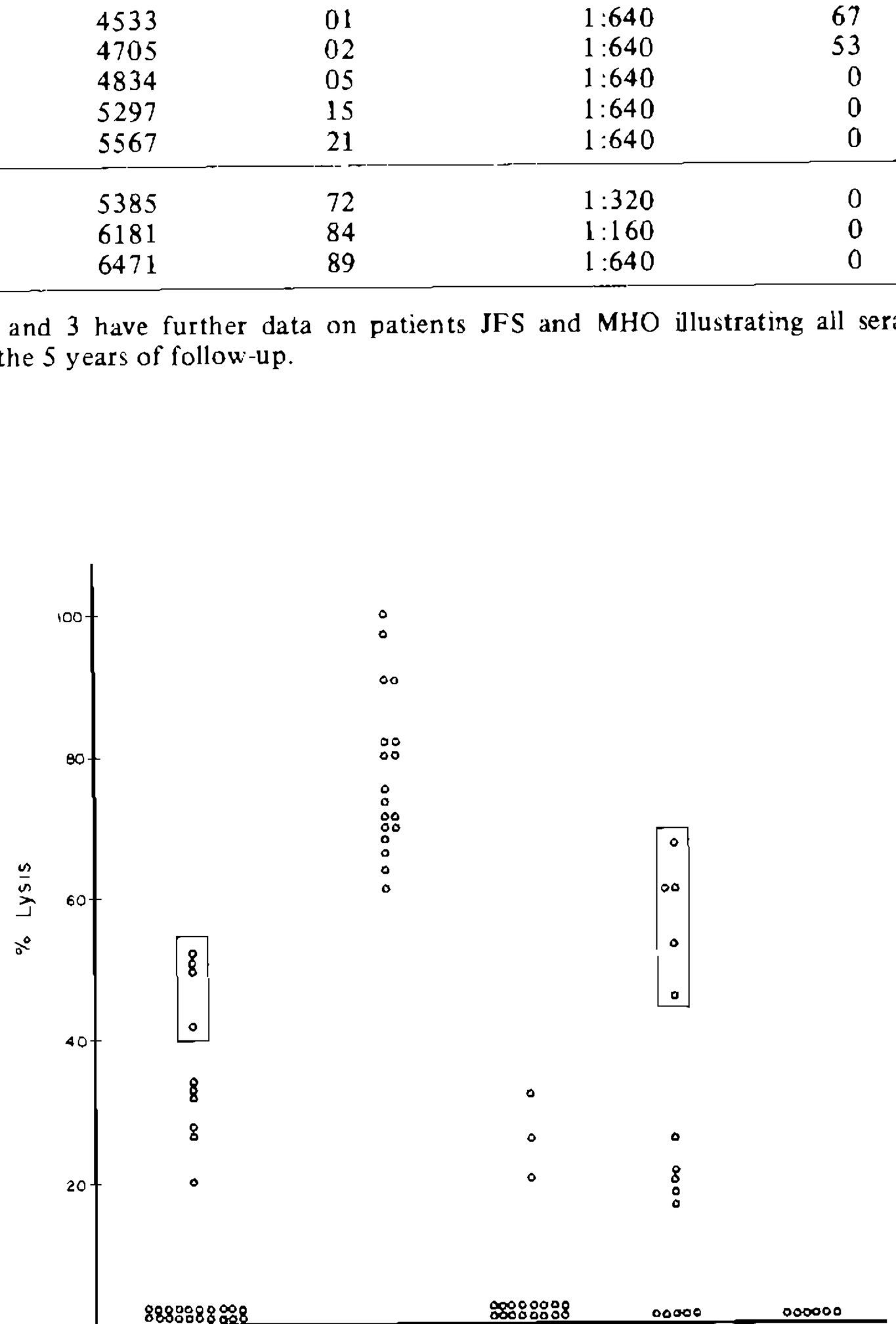

Fig. 2: percent of trypomastigote lysis induced by complement and individual sera from treated patients with Chagas' disease, considered either a "therapeutic failure" (IFR), or "cured?" (dissociated, Group C), all of them with high titers in the conventional serology.

Inside the rectangle there are lytic sera obtained at the year of treatment (JFS, MHO). Patients VAC, ACJ were firstly used two or more years after treatment. Observe that most sera of the dissociated patients were not lytic or had a borderline activity except in the year of treatment. IFR was treated several times being very representative of the uncured group with high lytic activity in all sera tested.

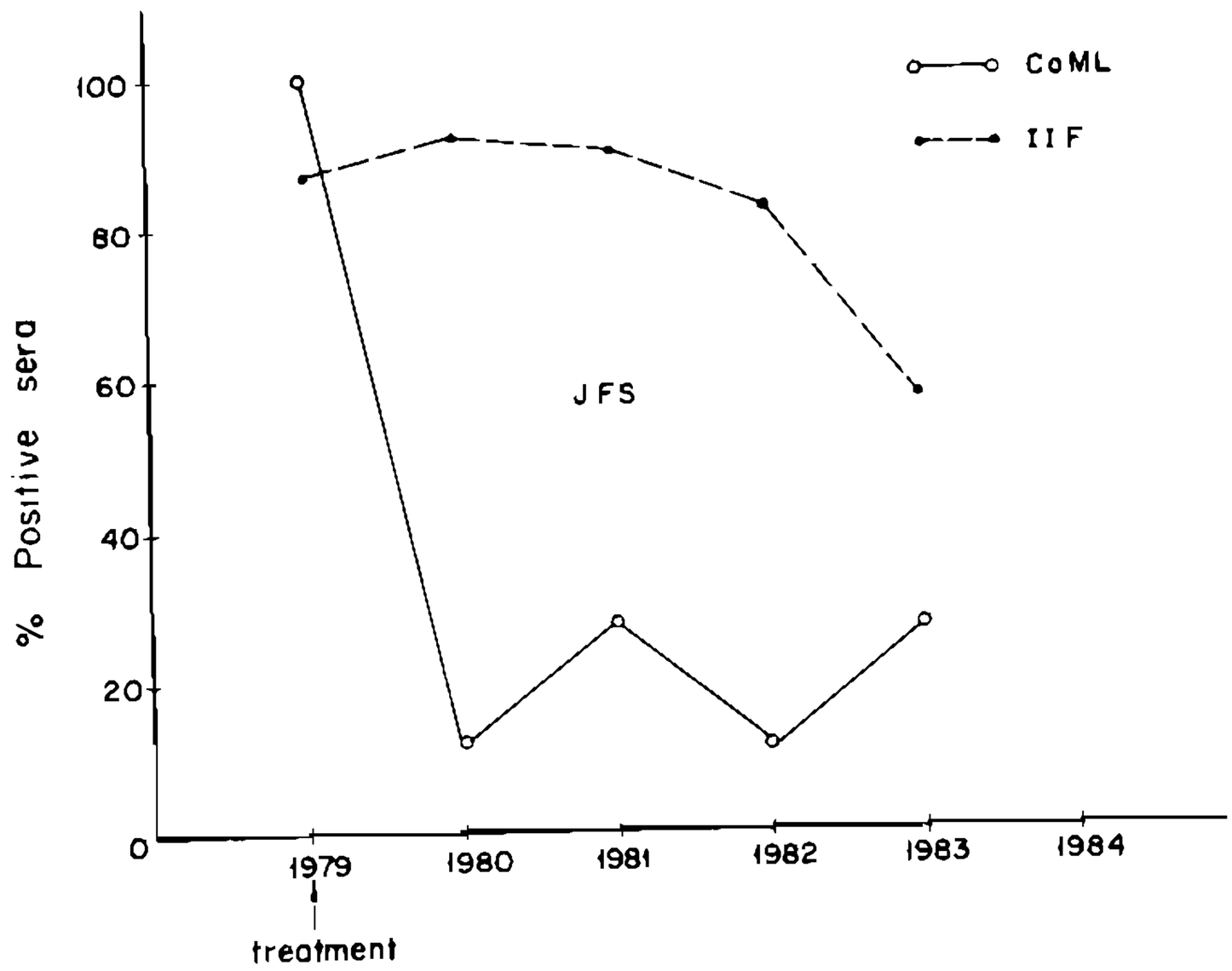

Fig. 3: evolution of serology performed by means of complement-mediated antibody dependent lysis (CoML) and conventional indirect immunofluorescence (IIF) in one chagasic patient during four years after specific treatment with a benznidazol-derivative.

Absence of ALBA on the "dissociated" group detected by ADCC, phagocytosis and neutralization tests

A total of 20 CoML negative sera from several patients of the treated "dissociated" group have been used in other functional tests against living bloodstream Try, namely, antibody-dependent cell mediated citotoxicity (ADCC), phagocytosis and serum neutralizing activity (SNA) as described in our enclosed review (Krettli). Two control groups of sera have been tested in paralel, i.e., from untreated chagasic patients as well as from treated "uncured" both presenting positive CoML and CS. It was found that only the CoML positive sera participated in the killing of *T. cruzi* induced by ADCC or SNA as well as in the increased phagocytosis by macrophage cells *in vitro*. Conversely, sera with CoML negative but CS positive, from the dissociated groups, behaved like normal sera from non-chagasic groups in the three tests, regardless of the presence of CS antibodies (Lima-Martins, Krettli & Brener, 1983; 1985; Lages Silva et al., 1984; Krettli et al., 1984). Therefore, patients with negative CoML are indeed lacking functional antibodies detected by several different methods *in vitro* or *in vivo* provided that the target *T. cruzi* cell used for their detection is the intact living Try.

ALBA antibodies discriminate high molecular weight polypeptides on the parasite surface

Sera from treated or untreated chagasic patients previously defined according to a long-term serological follow-up (CoML, IIF and CFR) were used in immunoprecipitation tests performed with surface labelled (I^{131}) *T. cruzi* antigens (Martins et al., 1985). The question was to define the antigen discriminated by ALBA using either CoML positive or negative serum. Among various patients there has been included our JFS patient previously illustrated (Fig. 2, 3; Table IV). His sera were tested in parallel, on the same experiment and in the same gel, being respectively CoML negative (one from 1979) or positive (one from 1982 and one from 1983). Sera which displayed ALBA positive recognized high molecular weight polypeptides (Mr 105, 120, 160); however, sera without lytic antibodies either lost or had a significantly reduced activity against those polypeptides (Martins, 1985). She has also immunoprecipitated surface antigens of epimastigotes with the various groups of sera and the pattern of glycoproteins, in this case, was similar regardless of the result of CoML. These data compared with similar studies also performed by Martins with sera from mice chronically infected (CoML positive) or immunized (CoML negative), show that the 160 Mr polypeptide seems to be the defined antigen against which the ALBA are direct. This subject and its implication were further discussed in our enclosed review.

Are the parasitological tests negative in patients lacking ALBA?

Although our results on chagasic patients points out to the conclusion that the lack of ALBA is an indicator of cure of the infection direct parasitological evidence that the parasite was indeed eliminated. Although knowing the actual limitations of the available methods we have chosen the hemoculture technique modified (Chiari et al., 1979) to disclose circulating parasites. This hemoculture test was performed in three groups of patients, treated or not and, so far, the preliminary data are very encouraging. None of the 11 patients studied once (and 6 repeated 2-4 times) from the "dissociated" group has given

a positive hemoculture (Galvão et al., 1984). In 12 treated, but uncured patients (therapeutic failure — Group A), 2 gave positive hemocultures (17%), whereas in the untreated patients 12 out of 26 (46%) had circulating BTry detected by the hemoculture method. Only the exaustive repetition of parasitological test, will allow to conclude whether the patients of the CoML negative "dissociated" group are indeed cured.

A group of 9 patients treated with benznidazol at the acute phase (A) and of 3 at the recent chronic phase (RC) i.e. 2-4 years after the acute phase were studied by Shikanai-Yassuda et al. (1984) by xenodiagnosis, CoML and CS, performed 1, 5 or 9 years after treatment. The authors only found positive xenodiagnosis in patients with positive CoML (2A and 1RC). They also stress the need of long follow-ups to establish the importance of the CoML in the control of cure.

The phenomenon of spontaneous cure in Chagas' disease is not known and there are evidences that Try may be detected for more than 50 years as demonstrated in the historical case of Berenice, the first case of human infection described by Prof. Chagas in 1909 and again studied by Salgado et al. (1962). Recent data on opossums *(Didelphis marsupialis)* experimentally infected with the Y strain suggests towards spontaneous cure of an animal (Tomaz et al., 1984). After a short period of patent parasitemia and positive IIF it became parasitologically negative (11 xenodiagnosis done) as well as serologically negative, for 8 consecutive months. The CoML was also negative being intensively positive in all the other animals studied. Although the above discussed data support our hypothesis that a negative CoML means absence of *T. cruzi* infection, the low positivity of the available parasitological methods complicates our evaluation. This difficulty will be hopefully overcome by new molecular biology techniques specially the recombinant DNA used as probes for parasite detection and favoured by the repetitive DNA sequences in the kinetoplast DNA of *T. cruzi.* The work developed now by several groups (see the enclosed chapter by Morel) is one of the most exciting data on the present research of Chagas' disease. We look forwards to the DNA probes hopping that, when available, they will uncover *T. cruzi* infections in all but in the "cured" as well as in our "dissociated" groups.

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