CHRONIC MYOCARDIAL DAMAGE IN EXPERIMENTAL T. cruzi INFECTION OF A NEW WORLD PRIMATE. CEBUS sp. MONKEY

Carlos Alberto FALASCA, M. D.; Monica GILL, M. D., Daniel GRANA, V. M., Elena GOMEZ, Jorge ZOPPI, M. D. & Eduardo MARESO, M. D.

SUMMARY

Eighteen Cebus apella monkeys, (juvenile and adult of both sexes) were inoculated five years ago, with three Trypanosoma cruzi strains (CA1, n = 10; Colombian, n = 4 and Tulahuen, n = 4), either by conjunctival or intraperitoneal route, once or repeatedly. Parasitological, hematological, serological, enzymatic, radiographic, electro and echocardiographic findings have been previously published and they are similar to those observed in human pathology. The most frequent electrocardiographic alteration was right branch bundle block.

Six animals, chosen at random, were sacrificed. Those sacrificed 20 to 25 months post-first inoculation showed focal accumuli of leukocytes with myocytolysis. Foci of diffuse interstitial fibrosis with mild infiltrate of leukocytes among fibers were observed in the animals sacrificed 36 to 47 months post-inoculation. No parasites were seen. The lesions were more prominent in the ventricular walls and the septum. The fact that the infiltrates were predominant in the animals sacrificed at a shorter time after first inoculation and that fibrosis was more severe in those sacrificed at a longer time suggests that there is a progression of the infiltrative lesions to fibrosis, with a leukocytic activity indicative of a chronic phase.

These lesions are similar to those described in human chronic Chagas' disease. This would demonstrate that this model is useful in evaluating a progress in the knowledge of the pathogenesis which is still a controversial issue, immunology, immunogeneses and chemotherapeutic agents of the chronic and indeterminate phases of this disease.

KEY WORDS: Cebus monkey; Chagas' disease; Pathology.

INTRODUCTION

Chagas' disease or American trypanosomiasis is a zoonosis, restricted to the American continent, caused by transmission of the protozoon Trypanosoma cruzi by triatomine bugs. It can also be transmitted by alternative mechanisms such as blood transfusion, congenital transmission, laboratory accidental infection, organ transplantation and the oral route.

This investigation received support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Disease (ID 790122), and by the Universidad del Salvador, Buenos Aires, Argentina.

Address for correspondence: Dr. Carlos A. Falasca, Instituto Latinoamericano de Investigaciones Medicas Universidad del Salvador (IILAMUS) Tucuman 1845 (11868) Buenos Aires, Argentina, tel.: 43 2935/812 9846 — Tlx.: 18660 UNIVDELBAL DELPHAR.
An efficient study of the pathogenesis of the indeterminate and chronic phases becomes difficult due to the slow evolution of Chagas' disease\textsuperscript{11}.

These difficulties have been accentuated "due to the lack of suitable animal models for chronic Chagas'disease, which would produce lesions resembling those found in human"\textsuperscript{17}.

The diversification of the research objectives and of the animal models chosen by the different research groups, makes it difficult to develop and reproduce, the electrocardiographic and pathological patterns of this disease. The different animal models under study are: rate\textsuperscript{28}, mouse\textsuperscript{29}, dog\textsuperscript{30} and rabbit\textsuperscript{31}, in search of the ideal experimental model\textsuperscript{1}.

Chagas' disease pathogenesis remains without a clear explanation although different mechanisms: immunological\textsuperscript{12, 27, 32}, neurogenic\textsuperscript{21}, hypoxemic\textsuperscript{20, 34} have been proposed.

As experimental Chagas'disease has been studied in Cebus sp monkeys by other authors without producing clear results\textsuperscript{6, 13, 24, 35}, the objective of the present work is to describe myocardial damage observed, after a five-year follow-up, in the Cebus apella monkey, a New World primate, native to the Paraguayan Chaco, experimentally infected with different T. cruzi strains.

Partial parasitological, immunological, electrocardiographic and echocardiographic studies have been previously reported \textsuperscript{14\textsuperscript{15}}.

The results show that this monkey is a suitable model of chronic Chagas'disease.

\textbf{MATERIAL AND METHODS}

Fifty-three Cebus apella monkeys, with normal electrocardiograms, echocardiograms and specific serology for Chagas'disease, were selected from a breeding and rearing outdoor colony. They were kept in captivity in the indoor colony in individual cages with free water provision and feeding was based on a standard pellet diet (25% protein, 3% lipids) prepared by Cargill (Buenos Aires, Argentina), supplemented with fresh fruit twice a week.

The animals were divided into four groups, one control and three infected. The age, sex, weight, parasite strain, route, and dose of inoculum and number of inoculations carried out are detailed in the summary of the experimental design (Table 1).

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{MATERIAL AND METHODS} & \textbf{STRAIN} & \textbf{CA 1} & \textbf{COLOMBIAN} & \textbf{TULAHUEN} & \textbf{CONTROL} \\
\hline
\textbf{Number of animals} & & 10 & 4 & 4 & 35 \\
\hline
\textbf{Sex} & & male & 2 male - 2 female & male & male \\
\hline
\textbf{Estimated age at first inoculation (years)} & & 6 - 10 & 1,2 - 3 & 4 - 4,5 & 5 - 9 \\
\hline
\textbf{Weight (q)} & & 2110 - 3320 & 940 - 1800 & 1660 - 1950 & 2250 - 2570 \\
\hline
\textbf{Date of first inoculation} & & 06 - 80 , 07 - 81 & 09 - 82 , 10 - 82 & 11 - 82 , 12 - 82 & --- \\
\hline
\textbf{Number of inoculations at 06 - 84} & & 1 / 2 & 17 / 18 & 10 / 11 & --- \\
\hline
\textbf{Number of T. cruzi (each inoculation)} & & 4 x 10\textsuperscript{6} to 1 x 10\textsuperscript{6} & 3 x 10\textsuperscript{6} & 3 x 10\textsuperscript{6} & --- \\
\hline
\textbf{Route} & & conjunctival & i.p. & i.p. & --- \\
\hline
\end{tabular}
\caption{Experimental Design}
\end{table}
The 10 adult monkeys receiving the CA1 strain were inoculated with the parasite's metacyclic forms by the conjunctival route. Three received one inoculation of $4 \times 10^6$ parasites; four received one inoculation of $1 \times 10^6$ parasites; and the last three received two inoculations, the first of $4 \times 10^5$ parasites and the second of $1 \times 10^6$ parasites one year later.

The two other inoculated groups (receiving the Colombian and Tulahuen strains) were repeatedly inoculated with $3 \times 10^6$ blood forms of the parasite by the intraperitoneal route — a route intended to produce better absorption of the parasite. The four young males receiving the Tulahuen strain were inoculated 10 or 11 times at intervals ranging from few days to 30 weeks and the four juvenile inoculated with the Colombian strain were inoculated 18 or 19 times at intervals ranging from 3 to 24 weeks. The periodic reinoculations were performed in order to approximate conditions found by people living in endemic areas, where the periods of natural reinfection vary.

The follow-up of the animals of the control group and the infected ones, along the course of the natural evolution, was performed by means of the control of parasitemia (fresh-drop, Strouts method\textsuperscript{11} and/or xenodiagnosis\textsuperscript{8} specific serology (indirect hemagglutination — IHB (Cellognost, Chagas Behringwerke) and enzy-moimmunoassay — ELISA 37), hematological parameters, plasmatic proteins and electrocardiogram. Xenodiagnosis was carried out with third nymphal stage Triatoma infestans, using 4 boxes containing 10 bugs each, that were examined 30 and 60 days afterwards.

The electrocardiograms were recorded using a Fukuda FSC-7100 monitor at a speed of 25 a 50 mm/sec. The animals were handled under 10 mg/kg/w ketaminehydrochloride anesthesia (Ketalar, Parke Davis, Buenos Aires, Argentina). The precordial leads used were V1 to V6 as in human, and V1R and V3R in order to obtain a better evaluation of the right cavities.

These studies were carried out once a week during the first three months of the infection and then twice a month during the first two years. At present, they are being performed once a month.

In order to carry out the objective of the present work, six animal chosen at random, from those inoculated that showed electrocardiographic disturbances were sacrificed\textsuperscript{37}. Four animals of the control group were sacrificed with the same purpose.

The whole heart and samples of intestine, liver, spleen, skeletal muscle and kidneys were collected. The heart was immediately removed, washed in 0.9% saline and fixed in Zamboni's liquid\textsuperscript{38}.

The heart was cut according to Olsen's technique\textsuperscript{38}. The material, 3 tissue samples of each ventricle and auricle, as embedded in paraffin after previous dehydration increasing alcohols and cleared in benzene; the sections were stained with hematoxylin-eosin, Masson's trichromatic and Movat's pentachrome and examined under light microscope.

By means of serial sections, the following areas were studied: the union of a superior cava vein (SCV) and the right auricle (RA); a search of the sinus node, and the portion of the interventricular septum corresponding to the septal flap of the tricuspid, a search of the atrioventricular node and the His bundle, were attempted.

RESULTS

General Comments

The follow-up of the control group showed no alterations in the different parameters studied. Neither gross nor microscopic cardiac lesions were observed in the control animals sacrificed.

During the acute phase, positive parasitemia was detected in all the infected animals either by the fresh drop, the Strout's method or xenodiagnosis. The period in which parasitemia started to become negative ranged from 5 to 60 weeks in the animals infected with CA1 strain, 18 to 54 weeks in those with the Colombian strain and 46 to 49 in those with the Tulahuen strain.

The highest titers for chagasic specific serology were 1/128 and 1/256 according to the T. cruzi strain utilized\textsuperscript{15}. They started to decrease
two and a half years after infection. At the moment of the sacrifice, all the animals had electrocardiographic patterns compatible with Chagas' disease.

Neither electrocardiographic alterations nor spontaneous deaths were recorded during acute phase, or immediately to inoculations.

At present, all the infected monkeys show electrocardiographic (Table 2)\textsuperscript{17}, and echocardiographic\textsuperscript{19} disturbances, which remained unmodified during the follow-up.

The electrocardiographic patterns in the group of infected animals resemble those described in human chagasic cardiomyopathy in the chronic phase.\textsuperscript{19} In the six sacrificed monkeys a right bundle branch block was detected in the three that had been inoculated with the CA1 strain. Left anterior hemiblock was observed in one monkey inoculated with CA1 strain, and in one with Colombian strain\textsuperscript{12}. In the ones with Tulahuen strain an intermittent right bundle branch block was detected. The other monkey inoculated with the Colombian strain exhibited repolarization disturbances.

**Morphological finding:**

**Control group:**

From the anatomical point of view, the “in situ” gross study of the heart of the primate, showed a vertical localization in relation to the longitudinal axis, similar to that of the slender human.

From the microscopic point of view the cardiac morphology was similar to that described in humans, both in the contractile tissue and the conduction system.

**Infected animals:**

**Gross anatomy**

In the animals sacrificed the heart was slightly enlarged and flabby with dilatation of the right side chambers (Fig. 1).

Thinning of the apical region of the left ventricle (apical aneurysm) was not found. Neither macroscopic nor microscopic signs of chronic cardiac failure were found.

**Table 2**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Monkey</th>
<th>RD</th>
<th>LVO</th>
<th>RVH</th>
<th>LAH</th>
<th>II RBB</th>
<th>ASF</th>
<th>RBBB</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA1 (n=10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>36</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>68</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>76</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>82</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>84</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>104</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>116</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA1 (n=10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>125</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>173</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>313</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colombian (n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>308</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>399</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tulahuen (n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>190</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>233</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>386</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>391</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **Sacrificed**
- **RD** = Repolarization disturbances
- **LVO** = Left ventricular overload
- **RVH** = Right ventricular hypertrophy
- **LAH** = Left anterior hemiblock
- **II RBB** = Intermittent right bundle branch block
- **ASF** = Anteroseptal fibrosis
- **RBBB** = Right bundle branch block
Light microscopy

A diffuse focal myocarditis with scattered fibrosis proportional to the time of infection was observed in all the layers of the heart and particularly in the ventricles. The coronary arteries showed no alterations. The mononuclear infiltrates of lymphocytes, plasmocytes and macrophages formed perivascular or interstitial accumuli around the fibers which showed myocytolysis (fig. 2).

These infiltrates were also observed in neurons and fibers of the parasympathetic nervous plexus of the interauricular septum. In some cases the nerves were completely infiltrated (fig. 3).

Another conspicuous feature was the diffuse focal fibrosis that was interstitial, perivascular or of substitution. This lesion was predominantly observed in the interventricular septum and the ventricular walls (Fig. 4).

In the conduction system, the specialized cells showed foci of myocytolysis surrounded by mild infiltrates. In the perinodal zone, a more severe infiltration was observed especially in the pericardium around the sinus node (Fig. 5 to 7). A mild fibrosis was detected in the atrioventricular node and in the His bundle (fig. 8).
Fig. 3 — Neuron cells of the ganglia of the interauricular wall surrounded by infiltrates of lymphocytes in a monkey 21 months after inoculation with Colombian strain (HE 400x).

Fig. 4 — Septum in a monkey 58 months after first inoculation with CA1 strain in which a marked interstitial fibrosis and scarce mononuclear elements in the fibrotic tissue are observed (Masson's trichromic 100x).

Fig. 5 — Sinus node of a monkey 53 months after first inoculation with CA1 strain presenting slight infiltrates among the specialized fibers (HE 40x).

Foci of severe myocardial fibrosis, either interstitial or of substitution, were the image more commonly found in the animals sacrificed 36 to 47 months post-infection whereas the principal characteristic observed in those sacrificed 21 to 25 months after first inoculation was the presence of interfibrillar and perivascular infiltrates with foci of myocytolysis.
This fact would suggest that there is a progression to fibrosis of the infiltrative lesions observed in the animals sacrificed sooner after first inoculation. On the other hand, mild mononuclear infiltrates among the myocardial fibers and foci of myocytolysis could be detected together with an important septal and ventricular fibrosis.

The ventricular myocardial cells showed enlarged nuclei and fibers, a sign of myocardial hypertrrophy.

**DISCUSSION**

The development of an animal model that resemble human Chagas’ disease has been a priority of the research plan devised by the RSG-SWG of the TDR of WHO.

The main objectives in this area, are to improve understanding of the immunopathogenesis of chronic lesions, the trial of new drugs and to develop vaccines against this disease.

Most experimental infections have been carried out in mice, rats and dogs, which are susceptible to *T. cruzi* infection. However the observations in susceptible hosts are usually limited to the early acute phase of the infection, probably due to the high mortality rate ensured by parasite inoculation. On the other hand, the *T. cruzi* experimental infection in mice does not reproduce either the distribution or the extent of the pannmyocardites observed in man. In contrast to mice, rats and dogs, rabbits and primates are apparently more resistant to *T. cruzi* infection. The course of experimental Chagas’ disease in adult *Cebus* sp. monkeys has been previously studied but with not very clear results.

These experimental studies have aimed at different aspects (clinical, parasitological, epidemiological, immunological) and have been carried out using different animal species, routes of inoculation, strain of parasites, length of infection, follow-up and method of evaluation difficulting the interpretation of them.
An animal model of human disease is a "living organism with an inherited, naturally acquired or induced by pathological process that

...in one or more respects, closely resembles the same phenomenon in man...". That is, it should accurately reproduce the disease or lesion under study.

The results obtained in this work, utilizing a different methodology to that used by other authors when experimentally infecting the Cebus monkey are very interesting specially those concerning the histopathological and clinical aspects since they resemble human chronic chagasic pathology.

During the acute phase, monkeys inoculated with Tulahuen strain showed positive parasitemia detected either by the fresh drop or the Strout method until week 8-30. In those infected with the Colombian or CA1 strain, the parasitemia could be detected only by xenodiagnosis and until weeks 39-54, respectively. This agrees with findings in man in the acute phase. Positive serology was observed from week 3 on. Neither electrocardiographic alterations nor spo...
neous deaths were recorded during this phase. These results would suggest that the morbidity observed in the course of the chronic phase in this model, as in humans, is not related to parasitemia. The electrocardiographic findings were similar to those described in humans during the chronic phase of the disease.

The histological sections of the heart showed focal myocarditis distributed in all the cavities, specially in the septum and free ventricular wall.

Mild to moderate focal infiltrates, formed by histiocytes, plasmocytes and lymphocytes, as well as myocyteolysis of the myocardial fibers, were evident. Focal and diffuse myocardial fibrosis, progressively increased, predominantly in septum and ventricles, as the time of infection augmented (36 and 46 months post-first inoculation) and evident sign of lesional evolution and chronicity, supported by the presence of activated fibroblast among the myocardial fibers.

It was possible to detect an hypertrophic response of the cardiac muscular fiber and substitution and interfibrilar fibrosis.

The focal nature of the myocardial lesion would be an indirect evidence of the microcirculatory compromise in this experimental model.

The inflammatory lesions were not related to the presence of the parasite, a fact reported by VIANNA in 1911, and that represents one of the characteristics of the disease. No amastigotes were found in the serial section study. Although the bulk of evidence indicates that intracellular parasites play a negligible role in the pathogenesis of Chagas' disease, recent papers suggest that intracellular parasitism might be more frequent than previously thought.

The lesions previously described, are similar to those usually found in human chronic Chagas' disease and that were described by ZILTON A. ANDRADE in humans and that are worthy to remember: "1) the cellular infiltrate composed of macrophages and lymphoid cells, tends to accumulate focally in areas where the local myocardial fibers show varying degrees of degenerative changes; 2) fibrosis appears as an outstanding feature, not only in focally dense areas but also in delicate and diffuse interstitial areas, sometimes involving each cardiac fiber; 3) congestion and edema are found throughout the myocardium; 4) the parasites are difficult to find in the histological sections."

In this model, the cardiac lesions were independent of the age, sex and weight of the animal and a rather long indeterminate phase, as it occurs in man who develops lesions years and even decades after being infected, and a chronic phase starting 2 and 3 years after infection, were observed.

The fact that the 100% of the animals survived the acute phase, and that histopathological lesions were similar to those of man, would demonstrate the extrapolation to the conditions and mechanism of human natural infection.

The animals infected with the Colombian and Tulahuen strains, repeatedly and using the i.p. route and a larger number of parasites than with the CA1 strain, showed histopathological lesions, in a shorter time (11-18 months post infection, Colombian and Tulahuen strains) than those receiving one or two inoculations (36-47 months post-infection, CA1 strain).

CONCLUSIONS

The Cebus apella monkey, native to an endemic area, and reproducing well in captivity, would be an experimental model suitable for the study of chronic Chagas' disease, since it reproduces the human electrocardiographical and histopathological alterations, in a short-time after experimental infection.

The use of this primate will permit us to progress in the knowledge of the pathogenesis and immunopathology of both, the indeterminate and chronic phase of this disease. It will also permit us to evaluate new immunogenic and chemotherapeutic agents acting in these stages.

This pathology, still unknown and difficult to treat, continues to be, as Chagas said, one of the most important and serious medico-social problems in Latinamerican rural areas. Furthermore, at present, it is also a serious problem in
the urban zones since the frequency of this pathology increases, due to the internal migrations and transfusional transmission by non detected chagasic donors.

**RESUMO**

Lesões miocárdicas crônicas na infecção experimental pelo *T. cruzi* no macaco (Cebus apella).

Dezoito macacos *Cebus apella* (jovens e adultos de ambos os sexos) foram inoculados há 5 anos atrás, com 3 cepas de *T. cruzi* (CA1, n = 10; Colombiana, n = 4 e Tulahuen, n = 4) seja por via conjuntival ou intraperitoneal, uma única vez ou repetidamente. Os achados parasitológicos, hematólogicos, sorológicos, enzimáticos, radiográficos, eletro e ecocardiográficos foram anteriormente publicados e são semelhantes àqueles vistos no homem. O achado eletrocardiográfico mais frequente foi o bloqueio do ramo direito.

Séis animais, escolhidos ao acaso, foram sacrificados. Aqueles sacrificados 20 a 25 meses após a primeira inoculação mostraram acúmulos focais de leucócitos com miocitólise. Focos de fibrose intersticial difusa com pequeno infiltrado de leucócitos entre as fibras foram observados em animais sacrificados 36 a 47 meses após a inoculação. Não foram encontrados parasitas. As lesões foram mais pronomeintras nas paredes ventriculares e no septo. O achado de infiltrados predominantemente, nos animais sacrificados em tempo mais curto em relação à primeira inoculação e a fibrose mais severa naqueles sacrificados após um tempo maior sugere que existe uma progressão das lesões infiltrativas até a fibrose, com atividade leucocítica indicativa de fase crónica.

Estas lesões são semelhantes àquelas descritas na doença de Chagas crônica humana. Este modelo, portanto, é útil na avaliação do progresso e conhecimento da patogênese da doença, assim como de sua imunologia, imunogenese e da ação da quimioterapia, tanto na sua fase crônica como indeterminada.

**ACKNOWLEDGMENT**

The authors wish to thank Prof. Dr. Zilton Andrade, Director Centro de Pesquisas Gonçalo Moniz, Brazil, for reviewing the manuscript and helpful criticism and the technical assistance of Ms. Nery Rolon and Mrs. Claudia Chiesa.

**REFERENCES**


Received for publication in 4/8/1989.