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Biochemical & histochemical changes relating to fibrosis following, infection with *Mycobacterium tuberculosis* in the guineapig

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Guineapigs infected with *M. tuberculosis were* studied for pammeters relating to fibrosis following infection. The infected animals were followed up to a period of 44 wk and the changes that occurred in the lung, liver and spleen were studied. Corresponding tissues from animals injected with bleomycin, an anti-mitotic drug which has the ability to produce pulmonary fibrosis, served as positive controls. Tissue collagen, elastin and hexosamines were estimated biochemically. The presence of granuloma and stainable collagen in paraffin sections of these tissues was also studied. Establishment of the infection was assessed bacteriologically by culturing the viable organisms from the spleen. It was observed that a self-limiting infection was established in the guineapigs and none of the animals died of the infection. In the infected animals, collagen, elastin and hexosamines showed an initial decrease followed by an increase. While the elastin and the hexosamine levels returned to the basal levels in all the three organs, collagen levels increased in the lung and were comparable to those of the bleomycin control. Collagen stainable by Van Gieson's method was found to be increased in the lung from the 4th wk onwards. The present report indicates the potential of adopting this system for studying mechanisms of fibrogenesis in tuberculous infection.

Key words Bleomycin - collagen - elastin - fibrosis - guineapig - hexosamine - tuberculosis

Tuberculosis, a granulomatous disease, is often characterised by extensive necrosis which can result in the massive destruction of the infected organ. It is known that when the disease is extensive, fibrosis can be seen even at the time of diagnosis'. When extensive destruction of tissue occurs, the consequent fibrosis leads to functional loss and disability.

Information regarding the changes relating to fibrogenesis in tuberculosis is scarce[2,3]. While it is difficult to delineate the events relating to fibrogenesis in human tuberculosis, this issue can be addressed in suitable animal models. It is possible to infect a number of experimental animals with *Mycobacterium tuberculosis.* However, it is known that among the various models, the pathogenesis of tuberculosis in the guineapig closely resembles that of the human disease[4]. In both human and guineapig tuberculous granulomata, the bacilli are predominantly extracellular. The distribution of the lesions in both species is similar. Therefore, the present investigation was initiated with the aim of infecting guineapigs with *M. tuberculosis* to assess some of the changes relating to fibrosis, biochemically and histochemically.

Bleomycin, an antimitotic drug is known to induce pulmonary fibrosis in experimental animals[5]. Therefore, guineapigs were treated with bleomycin and these served as a positive control for studying parameters relating to fibrosis.

Material & Methods

A total of 58 male guineapigs *(Cavia porcellus)* weighing about 250 g were obtained from the National Centre for Laboratory Animal Sciences, National Institute of Nutrition Campus, Hyderabad. *M. tuberculosis* H37Rv strain was obtained from the stock cultures maintained in Tuberculosis Research Centre, Chennai. The bacilli were suspended in saline and their number was adjusted using a Thoma bacterial counting chamber (Gallenkamp, England).

Six guineapigs were given bleomycin (Khandelwal Laboratories, Mumbai) 5 mg/kg body weight, ip twice weekly for 5 wk and sacrificed after a week of the last injection and lung, liver and spleen samples were collected, to serve as positive controls for fibrosis[6].

As most cases of tuberculosis occurring in India are of the post primary form, it was decided to sensitize 43 guineapigs by giving 10^3 live organisms ip. Six weeks after this sensitization, 37 animals were injected intramuscularly with an infecting dose of 10^7 organisms in 1 ml of saline[7]. Six sensitized but uninfected and nine unsensitized and uninfected guineapigs were sacrificed during the course of the study, to serve as normal controls.

Groups of three to six animals of the infected group were sacrificed at various time points from 4 to 44 wk following infection and the lung, liver and spleen from these guineapigs were collected. Bacteriological investigations were done with portions of the spleen collected at four weekly intervals from the 4th to the 20th wk and then at the 32nd and 44th wk after infection. Biochemical and histochemical investigations were done at 2 weekly intervals from the 4th to the 12th wk and then at 16th, 20th, 32nd and the 44th wk.

Bacteriological investigations : The whole spleen was removed aseptically and was divided into four portions. One portion was taken in a sterile bottle and was homogenised in 5 ml of normal saline and cultured for *M. tuberculosis* . Of the other three portions, one was transferred to a bottle containing about 10 ml of 10 per cent formal saline fixative, for histology. Collection of spleen for culture was not done with the uninfected control animals.

Biochemical investigations : Samples of lung and the liver were excised, washed by immersing in physiological saline. These were divided into three portions. One was immersed in formal saline for histological examination and the other two were processed for biochemical investigations after recording their wet weight.

Collagen and elastin contents of the tissues were estimated from their hydroxy proline content after acid hydrolysis[8]. Samples collected for elastin estimations were first subjected to three cycles of autoclaving at 15 lb psi for three h, and pelleting by centrifugation at 3000 rpm for 20 min to eliminate components other than elastin and then hydrolysed. Hydroxyproline content of the hydrolysates was assayed as described earlier[9] and suitable corrections were applied for dilution and the weight of tissue taken for hydrolysis. Collagen and elastin contents of the samples were obtained by multiplying their hydroxyproline values with a factor of 7.46 and 43.4 respectively[8].

The hexosamine content of the tissue hydrolysates was estimated using the method of Elson and Morgan[10] and the exact amount in each tissue was calculated by applying suitable corrections for dilution and weight of tissue taken for hydrolysis.

Histochemical investigations : For histological examination, specimens fixed overnight in formal saline were processed to obtain 5 micron thick paraffin sections. The sections were stained with haematoxylin and eosin (H&E) for the presence of the granuloma and by the Van Gieson method for collagen[11]. Collagen content in these sections was graded as either normal or as increased compared to the collagen content of sections obtained from the corresponding organs from normal controls.

Statistical methods : Comparisons between the controls and the test animals and between groups of test animals at different time points for each of the parameters studied were made using the Student's unpaired 't' test.

Results

Changes in the control animals :

Biochemical changes–As no significant differences were observed in the levels of collagen, elastin or hexosamine among the sensitized and unsensitized control guineapigs in all the three tissues (data not shown), the values from these two control groups were amalgamated for further comparisons. An increase in

the levels of elastin ($P < 0.05$) was observed in the lung of bleomycin treated animals when compared to the uninfected controls (Table I). An increase of about 33 per cent in the collagen and a decrease of about 32 per cent in the levels of hexosamine were observed in the lungs of bleomycin treated animals. However, due to high variability amongst the samples within the group, the levels were not significantly different compared to untreated controls. The contents of these three components were similar in the liver and spleen (Table I).

Histochemical changes– When the sections of lung, liver and spleen from bleomycin treated guineapigs were examined microscopically, it was found that all the lung sections were infiltrated by scattered collections of lymphocytes and macrophages. Similar collections of inflammatory cells were also seen in all the sections of the spleen. However, in the liver no inflammatory cells were seen in any of the sections.

When the amount of collagen staining in the lung was evaluated by comparison with the lungs from normal guineapigs, three of the six guineapigs treated with bleomycin had increased amount of fibrous tissue and the other three had normal amounts. This increase in collagen in the lung sections was observed mainly around the terminal bronchioles and blood vessels. Among the other organs examined, only one of the spleen sections and none of the liver sections showed increased stainable collagen.

Changes in the infected animals :

Establishment of infection–A portion of the spleen from the infected animals was cultured for *M. tuberculosis* at various time points from the 4th to the 44th wk. While *M. tuberculosis* could be grown from all the guineapigs at the fourth week, subsequently, the rate of culture positivity declined, though one or more guineapigs were still positive till the 44th wk.

Biochemical changes : *(i)* Collagen– The mean collagen contents of all the three tissues of the infected guineapigs are shown in Table II. In all the three tissues, the levels of collagen decreased initially till 10 wk after infection. From the 12th wk onwards, an increase in lung collagen levels was observed. The values from the 12th to the 44th wk were significantly elevated compared to the controls ($P < 0.05$) and were comparable with the level of 8.08 mg/g observed in the bleomycin treated animals (Table I). The collagen content of the liver also decreased significantly ($P < 0.05$) up to the tenth wk (except at eight wk) from 2.6 mg/g to 0.8 mg/g. However, the subsequent increase in the level of collagen was not significant. The spleen collagen also showed a similar initial decrease up to 6 wk followed by an increase.

(ii) Elastin and hexosamine– The mean elastin contents of all the three tissues of the infected guineapigs are shown in Table III. A significant decrease after infection ($P < 0.05$) was observed in all the three organs but only up to the sixth wk. Subsequently the elastin levels increased steadily to the preinfection levels by 12 wk.

The mean hexosamine levels in the lung, liver and spleen of infected animals (Table IV) also decreased

Table I. Levels of matrix components in control guineapigs

Organ/group	Collagen	Elastin	Hexosamine
Lung:			
Bleomycin (n=6)	8.08±0.4	2.22±0.1*	17.86±4.0
Uninfected (n=15)	6.23±1.3	1.43±0.4	25.81±6.7
Liver :			
Bleomycin	2.59±0.2	1.01±0.1	219.02±37.5
Uninfected	2.57±0.4	1.12±0.5	199.99±64.6
Spleen :			
Bleomycin	4.15±0.2	1.45±0.1	29.83±10.9
Uninfected	4.24±1.1	1.11±0.5	48.28±15.4

Values in mg/g tissue for collagen and elastin and in µg/g tissue for hexosamine are Mean±SE
* $P < 0.05$ as compared to the uninfected control

Table II. Levels of collagen in infected guineapigs

Weeks after infection	Number of animals	Lung	Liver	Spleen
4	4	3.33±0.2	1.41±0.2*	1.82±0.1*
6	4	1.67±0.3*	1.05±0.1*	1.53±0.2*
8	4	2.71±0.1*	4.27±1.8	2.65±0.3
10	4	2.27±0.2*	0.84±0.3*	2.48±0.4
12	4	10.85±1.8*	3.32±0.2	6.92±1.3*
16	4	8.73±2.3*	3.13±0.8	6.14±1.9
20	6	8.78±2.6*	3.25±0.6	6.06±1.5
32	4	6.76±1.3	1.68±1.0	3.92±0.3
44	3	5.61±0.6	2.51±0.1	2.71±0.2

Values in mg/g tissue are Mean±E
* $P < 0.05$ as compared to the uninfected control (shown in Table I)

Table III. Levels of elastin in infected guineapigs

Weeks after infection	Number of animals	Lung	Liver	Spleen
4	4	0.28±0.2*	0.46±0.1*	0.70±0.2*
6	4	0.11±0.1*	0.28±0.2*	0.29±0.1*
8	4	1.41±0.7	1.17±0.6	1.00±0.3
10	4	0.77±0.1	0.85±0.9	0.89±0.5
12	4	1.15±0.5	1.24±0.1	1.53±0.6
16	4	1.87±0.4	1.46±0.2	1.88±0.4
20	6	1.72±0.3	1.47±0.2	1.88±0.5
32	4	1.69±0.4	1.51±0.4	1.93±0.4
44	3	1.63±0.1	0.79±0.3	1.35±0.1

Values in mg/g tissue are Mean±SE
* P <0.05 as compared to the uninfected control (shown in Table I)

Table IV. Levels of hexosamine in infected guineapigs

Weeks after infection	Number of animals	Lung	Liver	Spleen
4	4	1.32±0.5*	7.91±3.1*	2.91±1.1*
6	4	2.96±1.1*	15.04±3.2*	3.82±0.9*
8	4	3.09±1.4	3.36±0.4*	1.53±0.2*
10	4	1.28±0.5*	8.71±1.7*	1.65±0.3*
12	4	2.71±1.3*	5.63±0.7*	4.68±1.3*
16	4	13.12±3.1*	68.18±21.3*	12.01±3.0*
20	6	13.71±3.1*	66.01±19.3*	11.24±2.5*
32	4	40.06±11.3	169.05±25.9	98.00±34.5
44	3	47.18±15.4	188.00±77.9	53.07±19.7

Values in µg/g tissue are Mean±SE
* P <0.05 as compared to the uninfected control (shown in Table I)

Table V. Number of animals with increased stainable collagen in the tissue sections at various time points after infection with *M. tuberculosis*

Weeks after infection	Total no.	Number of animals with increased collagen		
		Lung	Liver	Spleen
4	4	3	0	0
6	4	1	0	0
8	4	4	0	0
10	4	4	0	0
12	4	2	0	0
16	4	3	0	0
20	6	4	1	0
32	4	3	0	1
44	3	3	1	1

significantly (P <0.05) immediately following infection and remained low up to 20 wk after infection before rising to the levels found in the uninfected animals. The changes in hexosamine level in the liver were more marked compared to those in the spleen or in the lung.

Histochemical changes– In the animals infected with *M. tuberculosis,* granuloma was seen at four wk in all the three organs. The presence of moderate to extensive non necrotic granuloma was noted in ail the animals at ail time points in the lung and spleen. In the liver, there was only minimal granuloma formation and less than 50 per cent of the animals in each group showed histological changes suggestive of tuberculosis (data not shown).

Staining of the sections by the Van Gieson's method showed that there was increased deposition of collagen around the granuloma (Fig.), the terminal bronchioles and also around the blood vessels in the lung and this was seen from the fourth wk and persisted up to the 44th wk (Table V). Increased deposition of collagen was not seen in the liver or the spleen till the 20th wk and thereafter also was observed only in an occasional animal (Table V). In these two organs, deposition of collagen was found mainly around the granuloma. In the liver, it was seen occasionally in the periportal regions also.

Discussion

Tuberculosis heals by fibrosis which when in excess can lead to disability of the affected organ. The present study attempts to understand some of the biochemical and pathological changes relating to fibrosis following tuberculous infection using the guineapig model. Our results indicate that changes relating to fibrosis as evidenced by alterations in the levels of collagen both biochemically and histochemically are seen primarily in the lungs of both bieomycin treated guineapigs and the animals infected with *M. tuberculosis.*

Bieomycin, an antimitotic drug, can get accumulated in the lung and induce pulmonary fibrosis[5]. This drug has been used to induce fibrosis in various experimental animais[12-16]. The fibrogenic effect of this drug in the guineapig has not been documented so far in the literature. The data pertaining to the changes induced by bieomycin indicate that this drug has maximal effect

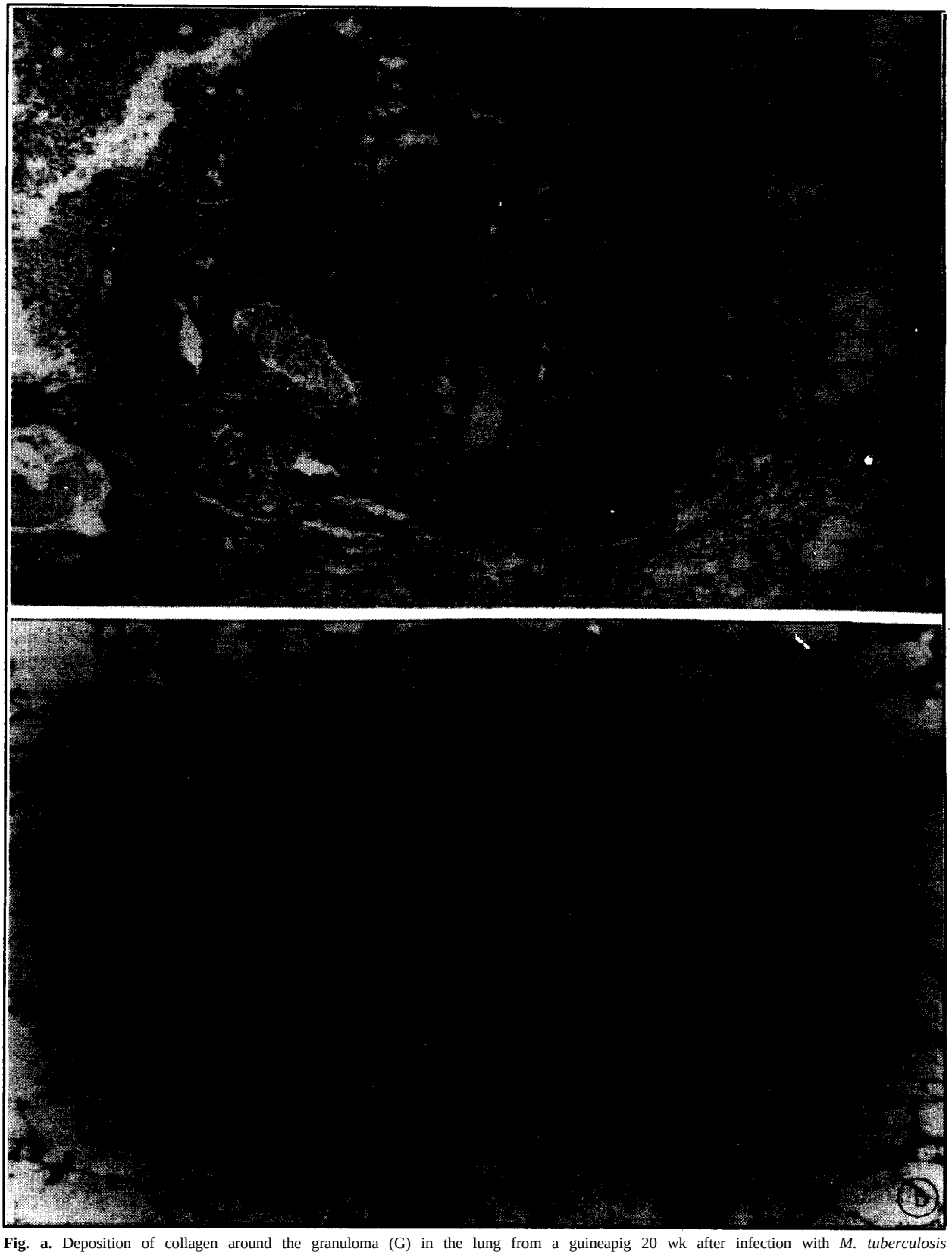

Fig. a. Deposition of collagen around the granuloma (G) in the lung from a guineapig 20 wk after infection with *M. tuberculosis* (Van Gieson stain, x 40). b. Section of lung from an uninfected guineapig (Van Gieson stain, x 70).

on the lung. However, the fibrotic changes observed are not as marked as those observed with other model systems[5,12-16]. It is possible that a higher dose and a longer treatment with bleomycin may induce a more pronounced effect in the guineapig model system.

Establishment of the infection with *M. tuberculosis* in the guineapigs was assessed by determining the presence of viable organisms in the spleen and the presence of tuberculous granuloma in the three organs. Results of the bacteriological examination reveal that the infection seen by the fourth week appears to be largely self-limiting as none of the infected animals died of the disease and fewer animals had viable organisms during the later periods of infection.

We have observed over a period of nearly 20 yr that the guineapigs used in our experiments have gradually acquired resistance to the regular strain of *M. tuberculosis* H37Rv (unpublished observations) and this could be the reason for a less virulent form of the disease without obvious caseation necrosis and no mortality in these guineapigs. It is likely that the use of other strains might alter the picture as has been shown in mice where strains of *M. tuberculosis* which had a lower virulence produced a more chronic pathology with formation of fibrosis[17].

Increased lung collagen and increased collagen synthesis in lungs have been demonstrated in several animal models[13-18]. However, in the lungs of the guineapigs infected with *M. tuberculosis*, there was a decrease in the collagen levels up to the first 10 wk, after infection, followed by an increase which persisted up to the end of the observation period. The initial decrease could be due to the release of matrix metalloproteinases which destroy certain types of collagen. It is known that *M. tuberculosis* and some of its major components like lipoarabinomannan can directly and indirectly release these enzymes from the granulomatous cells[19].

Histochemically, increased collagen could be seen in the lung of the guineapig almost throughout the period of study. This is similar to the findings of Marshall and coworkers[2] who have shown that neocollagenesis occurs in a granuloma induced by purified protein derivative from the second day onwards. Haschek *et a1* [20] observed in an experimental model of acute pulmonary fibrosis, a decrease in newly synthesized type III collagen together with an increase in synthesis of total collagen. Such a phenomenon could explain the differences observed between biochemically estimated total collagen and the histochemical staining in the initial phase of the infection. In the liver, the deposition of collagen found in the periportal region is similar to the findings reported from human studies[21].

The levels of elastin in the guineapig lung initially decreased and then increased significantly after the sixth week. Although new elastin formation was observed, biochemical estimation does not provide information about the quality of elastin formed e.g., its elasticity and fibril formation. On the other hand, it is possible that the elastin formed may be amorphous lacking elasticity. Further analysis is required to determine the quality of elastin formed.

Hexosamine levels varied considerably from animal to animal. It is likely that the varying amounts of congestion and stasis of blood during autopsy could have contributed to the variation. In spite of the huge variation, an initial decrease followed by normalisation to the preinfection level was observed with hexosamine also, but the levels started raising only after 20 wk following infection.

The results reported in this preliminary investigation indicate that the use of different strains of *M. tuberculosis* with varing virulence might establish a more suitable model for studying fibrogenesis following tuberculosis. Therapeutic manipulations for minimising the deleterious effects of fibrosis in the healing process of tuberculous lesion could then be attempted using such a model.

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