SERUM PROTEIN CHANGES IN EXPERIMENTAL TUBERCULOSIS AND IMMUNITY *

By M. Indira and M. Sirsi
(Pharmacology Laboratory, Indian Institute of Science, Bangalore-12)

Received on March 17, 1959

Abstract

The pattern of protein changes during the development of immunity after B. C. G. vaccination and during infection of these immunized animals have been compared with those occurring in non-immunized healthy guinea pigs infected with virulent tubercle bacilli and later treated with INH. The protein patterns were studied by the agar-gel electrophoretic technique.

The main features of the results obtained were: (1) in acute infection of normal guinea pigs, a fall was noticed in the albumin level and a rise in all the globulins with no alteration of the total proteins in the earlier phase of the infection; a steep fall in the albumin and increased rise in all the globulins with hyperproteinaemia in the later stages. Treatment with INH raised the lowered albumin and decreased the β globulin levels.

(2) After B. C. G. vaccination no alteration in the albumin concentration, but a slight fall in $\alpha 1$, $\alpha 2$ and β globulins and increase in the γ globulin associated with hypoproteinaemia was the early reaction. From the second month onwards, an increase in $\alpha 1$, $\alpha 2$ and β globulins associated with hyperproteinaemia was observed. During the development of tuberculosis in the immunized animals, $\alpha 1$ globulin remained at the immunized level; $\alpha 2$, β and γ globulins showed an increase, the latter to quite an appreciable extent. Significance of these changes in the individual components in relation to immunity and infection are discussed.

The various components of the serum proteins undergo very marked changes in tuberculosis. Both qualitative (Seibert and Nelson, 1942; Weimer and Moshin, 1953) and quantative changes in the Serum proteins (reviewed by Gutman, 1948) have been reported. The use of the moving-boundary type of electrophoresis as a very sensitive tool for analysis of tuberculous sera was recognized through the works of Leutscher (1941) and Seibert and her associates (1947). Of late, zone electrophoresis, in particular that of paper, has been widely adopted due to its simplicity, high resolution and reproducibility. The present investigation has been primarily confined to the adoption of the recently developed agar-gel electrophoresis (Giri, 1956, *a*, *b* and *c*) in the study of the serum protein changes during experimental tuberculosis and B. C. G. immunization in guinea pigs.

* Forms part of a thesis for which Associateship of the Indian Institute of Science has been awarded to Miss M. Indira.

Materials and Methods

Adult guinea pigs of both sexes weighing from 450—700g. were housed in separate cages and were fed the standard laboratory diet which consisted of leafy vegetables 40% ; wheat bran, 20% and soaked bengal gram, 40%. The diet was given *ad lib*. This diet has been found to be adequate for both growth and reproduction in our stock colony of animals.

All the animals were tested intradermally with 0.1 ml. of 1/10 dilution of Old Tuberculin (human) prior to the investigation. The reaction was considered positive if the diameter of the induration after 24 or 48 hours was 0.5 cms. or more. Only tuberculin negative animals were taken for investigation.

The organisms used were *Mycobacterium tuberculosis* $H_{37}R_v$ and B.C.G. strain obtained from B. C. G. Vaccine laboratory, Madras. Both the organisms were transferred from Petrick's solid medium to Youmans medium (Youmans and Karlson, 1947) as a surface culture prior to inoculation. The animals were inoculated with either of the organism intramuscularly in the thigh region with a Saline suspension (1mg. of bacilli per animal).

After allowing the virulent infection to progress for about eight weeks, treatment with isonicotinic acid hydrazide was started (30 mg/kg. body weight orally every day). The protein changes after B.C.G. vaccination are followed for a period of 4 months after which time the challenging infection with *Myco. tuberculosis* $H_{37}R_v$ (1 mg./animal) was administered. Further protein alterations during this infection of immunized animals were followed for a period of six months.

Blood for analysis was taken from the animal by cardiac puncture. Serum was prepared by centrifugation after the formation of the clot.

The total proteins were analyzed by the method of Lowry *et al* (1951) using the Folin-Cicalteau's phenol reagent.

The electrophoresis of the sera was done using the agar-gel electrophoresis (Giri, *a b* and *c*). 20 μl of serum was used for analysis. The electrophoretic patterns after the dyeing of the patterns were scanned by using a Photovolt Electronic Densitometer Model 525. The area under each peak of the densitometric curve was measured by using a planimeter.

The results are summarized in the tables I and II. Representative serum protein patterns obtained in :

(*a*) normal guinea pig,
(*b*) advanced stage of infection with virulert $H_{37}R_v$ bacilli,
(*c*) 2 moths after treatment of the above with isonictotinic acid hydrazide,
(*d*) 2 months after vaccination with B.C.G., and
(*e*) 1 month after a challenge infection of the immunized animal with virulent $H_{37}R_v$ bacilli,

are shown in Plates I, II, III, IV and V, respectively.

TABLE I

Serum protein changes during the course of infection and treatment with INH

Period after infection	Months after treatment	No. of animals	Albumin	$\alpha 1 + \alpha 1'$	$\alpha 2$	$\beta 1 + \beta 2$	γ	Total proteins gm.%
Normal	–	10	73.7	4.2	9.6	5.1	7.6	5.8
3 weeks	–	10	65.5	5.2	11.0	9.0	11.0	5.7
2 months	–	4D	33.7	9.5	12.5	16.6	28.6	8.0
2 months	–	6	54.2	6.4	12.0	15.4	15.4	7.4
3 months	1	6	50.5	11.9	11.6	16.5	13.3	6.9
4 months	2	6	58.8	12.4	11.5	12.1	12.4	6.9

Animals marked D died during the early part of the third month

All the protein values are expressed as percentage of the total area of densitometric curve and represent the mean values.

TABLE II

Serum protein changes during the course of immunization with *M. tuberculosis* ($H_{37}R_v$)

Period after immunization	Months after challenge	No. of animals	Albumin	$\alpha 1 + \alpha 1'$	$\alpha 2$	$\beta 1 + \beta 2$	γ	Total proteins gm.%
Normal	–	6	71.6	6.0	9.0	6.3	7.6	5.9
3 weeks	–	6	72.1	5.6	6.9	5.2	8.7	5.2
2 months	–	6	67.4	6.0	11.0	7.6	8.3	7.2
3 months	–	6	68.8	7.4	10.7	7.7	6.1	7.3
4 months	–	6	69.7	7.5	10.3	8.3	5.4	7.0
5 months	1	3A	57.3	12.0	12.4	6.3	11.6	7.5
5 months	–	3B	70.2	7.6	11.0	9.2	6.6	7.2
6 months	2	3A	59.3	8.8	12.7	7.7	12.1	7.3
6 months	–	3B	70.2	8.3	10.9	9.5	5.7	7.1

Group A was challenged with M. tuberculosis at the end of 4 months.

Group B was only B. C. G. immunized.

All the protein values are expressed as percentage of the total area of densitometric curve and represent the mean values.

Discussion

The lowered level of albumin during infection and a rise in its level during a successful treatment has been observed by all previous workers. Our findings also confirm these observations. During immunization, throughout the period of observation no significant fall was noticed. This may be due to the low dose of vaccine used (1 mg.) since Kanagami and Motomiya (1955) have reported that the fall in the level of albumin is proportional to the amount of B. C. G. given. Hudgings *et al* (1956) report a significant fall in its relative concentration four weeks after the inoculation of 3 mg. of B. C. G. per guinea pig. Haruo Kanagami (1952) on the other hand found the fall in albumin only during the first week after immunization and that the normal level was reached by the fourth week. In humans, following B. C. G. vaccination, Gilliland *et al* (1958) noticed a slight but significant fall in the albumin persisting even upto 18 weeks. An initial reduction in albumin occurred in the immunized animals challenged with a virulent organism and this did not progress further when observed at the end of the second month. This clearly indicates that resistance has been conferred and the progress of the diseases has been arrested by the immunizing dose of B. C. G. given.

Among the α-globulins, the α1-fraction gradually increased, a marked change being noticed during the second month after the infection. The change in the α2-globulin indicates a general increase during both the months of observation. Hudgins *et al* (1957) in a similar work, could not observe any significant change in the level of this fraction. A sharp increase has however been observed by them in rabbits infected with a virulent bovine strain and in humans by Baldwin and Iland (1953), Seibert and associates (1947) and Gutman (1948). They considered this fraction to be mainly derived from tissue destruction.

During treatment, both the α-globulins persist to be in the increased levels. This does not confirm the observations of Kazuko Machida (1948) who found that the treatment caused a lowering of the above said fraction.

In B. C. G. immunization, the α1-fraction commences to increase slightly from the third month onwards up to the experimental period. But Hudgins *et al* 1956 report a significant increase as early as four weeks after immunization. In the case of the α2-globulins, a decrease initially and later an increase which persists up to the fourth month, is noticed. Gilliland *et al* (1958) noticed no change in α1 but a slight and significant increase in α2 in humans following B. C. G. vaccination. Challenging infection in the immunized animals is seen to accentuate the production of α1 and α2 globulins earlier and during the second month a decrease in the α1 globulin was noticed. Meguro and Morikawa (1954) report that there is a temporary decrease of the α-fraction as a whole after a challenge infection. But in our observations, an increase in both the α-fractions is found during the first month after challenge.

Since antibodies related to tuberculosis could not be demonstrated till now in the α and β globulins these fractions were considered to be unimportant in acquired resistance. But Zitrin and Wasz-Hoellert (1957) have demonstrated that a fraction of tuberculosis sera containing α and β-globulins possessed growth inhibitory action on *M. tuberculosis, in vitro* and a protective action against a tubercular infection *in vivo*. It is known that after infection and treatment with chemotherapeutics, the animal is conferred with a certain degree of acquired resistance. Since α and β-globulins have thus been shown to possess anti-tubercular properties, it is possible that this resistance is partly explained by [i] the persistance of the high level of α1-globulin in INH treated, immunized and challenged animals and by [ii] the occurrence of an increased level of α2-globulins in infected and subsequently treated animals as also in B. C. G. immunized animals.

In the present study, it is noted that the β-globulins are affected earlier and to a great extent by the end of three weeks after infection. Hudgins *et al* [1957] however, report that the α1-fraction is the first to be significantly affected, and also Seibert and Nelson [1942] observed the same in rabbits infected with a virulent bovine strain. A further increase in the β-globulin during the second month is noticed, change being more severe in the animals which had the lowest albumin values. This has been observed by all the previously mentioned authors. Since the β-globulins are mostly lipoproteins, a conjecture has been made by Seibert and Nelson that the increase in this component in tuberculosis may be related to the lipoidal nature of the tubercle bacillus. During the immunization with B. C. G. after an initial decrease a tendency towards an increase is noticed throughout the experimental period.

The challenging dose given to some animals, initially produced a lowering in the β-globulins. But during the second month, an increase was noticed. This probably reflects to a certain extent on the degree of acquired resistance of the animals towards infection.

The increase in the γ-globulins was well noticed at the end of the first three weeks after infection in most of the animals and in all the animals in the second month. This is in conformity with the results of authors like Seibert and Nelson [1942] and Harrower *et al* [1957] who found only a delayed response in the γ-globulins. The large rise in this fraction cannot be traced only to the presence of antibodies. Bjorneboe [1943] found enormous increase in the γ-globulins, altogether disproportionate to the concentration of specific antibodies detected by quantitative methods. During the two months of treatment with INH, the level of this fraction is seen to be not much affected, though there was tendency in some animals towards a lowered level. This observation is in close agreement with that of Kazuko Machida [1948] who found that treatment with INH or streptomycin did not affect the level of γ-globuin. During immunization, the increase in this fraction is noted by the third week. This is probably due to the antibody response. But it is interesting to note that the

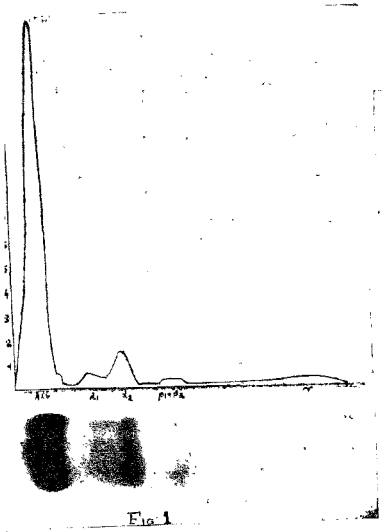

FIG I

PLATE I

An agar electrophoretic pattern of serum proteins of normal guinea pig

(20 μl of serum, 200 v, 6-7 m.a., Veronal buffer pH 8.6, I.S. 0.05 ; 4-5 hr. run)

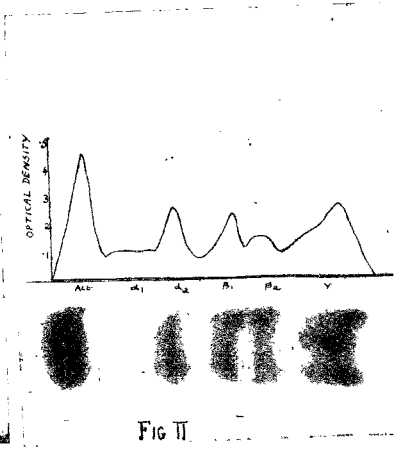

FIG II

PLATE II

(Advanced stage of infection with virulent tubercle bacilli, $H_{37}R_v$. Note the large decrease in the albumin and increase in all the globulins.

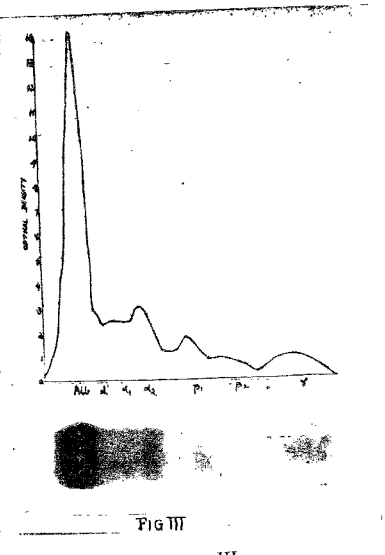

FIG III

PLATE III.

Two months after treatment with isonicotinic acid hydrazide (INH). The albumin is seen to be increased. The globulins are also at increased levels. The band indicated α_1^1 was found to separate on a few occasions. This has been incorported with α1 for evaluation.

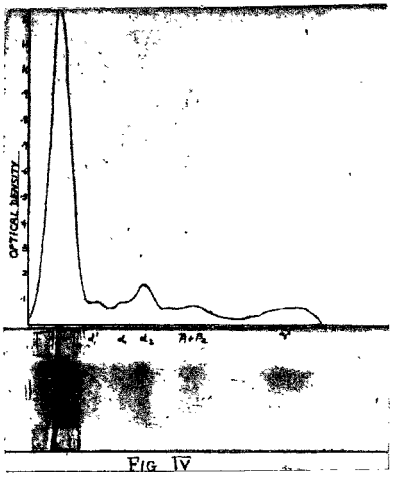

FIG IV

PLATE IV

Two months after vaccination with B. C. G. Note the slight increase in the β and γ globulins.

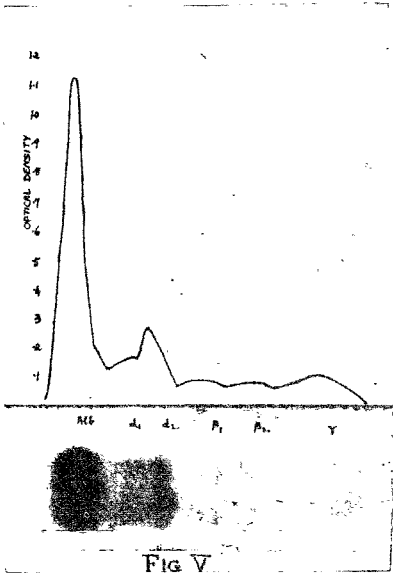

FIG V

PLATE V

One month after a challenging infection with virulent tubercle bacilli $H_{37}R_v$ to the B. C G vaccinated animal. Note the decrease in the albumin and the increase in $\alpha 2$ and γ globulins.

level is not maintained during the following months. Already two of the animals showed signs of decrease by the end of the second month alone. Its production is greatly stimulated during the challenging infection. The quantitative difference in γ-globulin production during a virulent [$H_{37}R_v$] and a non-virulent infection [BCG] is thus found to be well marked.

The present investigation indicates a severe hyper-proteinemia at the terminal stages and a moderate hyper-proteinemia during the initial stages of the disease. These results confirm those of Hudgins *et al* [*1957*], Haruo Kanagami [1952], Weimer *et al* [1954] Angulo Ortega [*1954*]. The treatment with INH did not significantly affect the proteinemia during the two months of observation. Ewerbeck and Wechselberg [1953] have reported similar results with regard to the effect after treatment with INH. During the immunization produced with B. C. G., an increase in the total proteins is noticed by the end of the second month and this is seen to persist without further increase in the animals even at the end of six months. A similar arrest of hyper-proteinemia is observed after the treatment of primary infection INH. The resistance conferred by B. C. G. vaccination can well be noticed, in that, no further increase in the total proteins was found after the challenging infection, whereas with the primary infection a progressive increase is a prominent feature.

REFERENCES

1. Angulo Ortega . . *Beitr. Klin. Tuberk.*, 1954, **3**, 229.
2. Baldwin and Iland . . *Am. Rev. Tuberc.*, 1953, **68**, 372.
3. Bjorneboe . . *Acta. Path. Microbiol., Scand.*, 1943, **92**, 415.
4. Ewerbeck and Wechselberg . . *Z. Kinderheilk*, 1953, **73**, 421.
5. Giri . . *J Indian Inst. Sci.*, 1956*a*, **38**, 190.
6. _____ . . *J. Lab. Clin. Med.*, 1956*b*, **48**, 775.
7. _____ . . *Naturwiss.*, 1956*c*, **43**, 36, 232, 226.
8. Gilliland, Stradling and Abdel Wahab *Brit. Med. J.*, 1958, *Jan.* 11, 87.
9. Gutman . . *Adv. Protein Chemistry*, **4**.
10. Harrower, Khan, Becker and Shabart. *Am. Rev. Tuberc.*, 1957, **76**, 892.
11. Haruo Kanagami . . *Science Repts.*, 1952, **C4**, 163.
12. Hudgins, Cummings and Patnode *Proc. Soc. Exp. Biol. Med.*, 1956, **92**, 75.
13. Hudgins and Patnode . . *Ibid.*, 1957, **95**, 181.
14. Isso and Marenzi . . *Pups. Centro. Invest. Tsiol., (Buenos Aires)* 1944, **8**, 168.
15. Kanagami and Motomiya . . *Sci. Repts. Research Inst., Tohuku Univ. Ser.* 1955, **C6**, 53.
16. Kazuko Machida . . *Nagoya Med. J., N.* 121, 1948, **66**, 578.

17. Leutscher .. *J. Clin. Invest.*, 1941, **20**, 47.
18. Lowry, Roseborough, Lewis Farr and Randall. *J. Biol. Chem.*, 1951, **193**, 265.
19. Meguro and Morikawa .. *Japan. J. Tuberc.*, 1954, **2**, 229.
20. Seibert and Nelson .. *J. Biol. Chem.*, 1942, **142**, 29.
21. Seibert, Seibert, Atno and Campbell. *J. Clin. Invest.*, 1947, **26**, 90.
22. Tiselius .. *Trans. Faraday Soc.*, 1937, **33**, 524.
23. Weimer, Redlich-Moshin, Boak, Bogen and Carpenter. *Am. Rev. Tuberc.*, 1954, **70**, 334.
24. Weimer and Moshin .. *Am. Rev. Tuberc.* 1953, **68**, 594.
25. Youmans and Karlson .. *Am. Rev. Tuberc.*, 1947, **55**, 529.
26. Zitrin and Wesz-Hoellert .. *Am. Rev. Tuberc.*, 1957, **76**, 256.