EXPERIMENTAL AMYLOIDOSIS IN MICE

A PATHO-SEROLOGICAL STUDY

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IN a previous publication (Tal and Laufer, 1960) it was found that there was no increase in the number of plasma cells in the spleen of mice injected with Freund's adjuvant or its components at the stage at which amyloid had been deposited. This does not, however, negate the importance of these cells in the early stages of amyloid formation (Ehrich, 1952). Thus it was found, by studying smears from splenic biopsies of mice injected at weekly intervals with Freund's adjuvant, that there was first a proliferation of plasma cells which, at a later stage, were transformed into " flame " cells containing material with the staining properties of amyloid. These cells eventually disintegrated and liberated the amyloid staining material (Zlotnick and Tal, 1960).

The purpose of the present study was : (1) to correlate the presence of plasma cells in smears of splenic biopsies, at various intervals after antigen injection, with the appearance of amyloid in histological sections ; (2) to determine whether amyloid possesses serological specificity to the antigen used in its production.

MATERIALS AND METHODS

Two groups each of 210 W-Swiss H line mice, inbred, 5 weeks old, were used in this experiment. Group I received weekly intramuscular injections of 0·3 ml. of adjuvant (50 ml. saline, 50 ml. Bayol F and 250 mg. heat killed *Mycobacterium tuberculosis*, type H37 RV var. *hominis*). Group II was treated in the same way except that the tubercle bacilli in the adjuvant were replaced by 250 mg. of a mixture of *Salmonella typhi* and *Salmonella paratyphi* A and B bacilli concentrated by centrifugation from a standard TAB vaccine.

Splenic biopsies were performed under ether anaesthesia before each injection. Smears were prepared, dried in air and stained with May-Grünwald-Giemsa, gentian violet, toluidine blue and congo red. Part of the last splenic biopsy was fixed in formalin for histological examination, and stained later with haematoxylin and eosin, congo red and gentian violet.

At the end of the 7th week, following the 6th injection, almost all animals showed amyloid deposits in the spleen. At this stage, 170 animals of each group were killed by decapitation. The blood of each group was pooled, serum prepared and kept at $-20°$ until used in the serological studies. The crude amyloid prepared from the spleens of these mice was used for serological studies and for immunization of rabbits.

The remaining mice, 40 from each group, were used for determining the time relationship between the appearance of agglutinins in the serum and in the amyloid supernatant fluid prepared from the spleens. As soon as the splenic biopsies revealed amyloid deposits, 10 animals of each group were killed by decapitation, and the remaining 30 were killed in groups of 10 at weekly intervals thereafter. The sera of each group of 10 animals were pooled and kept at $-20°$ until used. The sera and the crude amyloid prepared from the spleens of these mice were used for serological studies.

Preparation of crude amyloid.—The spleens were removed under sterile conditions, pooled, minced and passed gently through a wire mesh (Pikovski, Tal, Schlesinger and Margoliash, 1957) in order not to damage the cells and then added to 16 ml. of saline. The mixture was centrifuged at 2000 r.p.m. for 5 min. to eliminate the cells and other debris. In this way a cell free supernatant fluid was obtained and kept at −20° until used. Since later histological studies showed that almost all the spleens contained amyloid, this fluid, containing the crude amyloid, will be referred to as amyloid supernatant fluid (ASF). Cell free supernatant fluid was prepared in a similar way from the spleens of normal mice to serve as a control for the serological tests, and will be referred to as normal supernatant fluid (NSF).

Immunization of rabbits with ASF.—Rabbits were immunized with the amyloid supernatant fluid prepared from the spleens of mice after 6 injections of the respective antigens. To 10 ml. of the ASF, 10 ml. of Bayol-F and 50 mg. of tubercle bacilli were added as adjuvant and mixed in a Waring blender for 4 min. Three injections of 1 ml. of this mixture were given on alternate days into the muscles of both hind legs, each animal receiving a total of 6 ml. After an interval of one week, the rabbits were injected intraperitoneally with 5 ml. of the same supernatant fluid, but without the adjuvant. After 10 days, they were bled by cardiac puncture and their serum was kept at −20° until examination.

Serological studies.—The sera and ASF of the various groups of mice were tested for their agglutinating ability against TB antigen (Takahashi, 1962) and TAB. In addition, the ASF obtained after the 6th injection was tested by the agar double diffusion technique against the rabbit anti-ASF sera (Ouchterlony, 1958).

RESULTS

After the second injection of the mice with either saline-oil-TB mixture or saline-oil-TAB mixture, the splenic smears already revealed a marked proliferation of plasma cells, which are scanty in splenic smears of normal mice. After the third injection " flame " cells appeared and their number increased steadily with the number of injections, while the normal-appearing plasma cells decreased. Following the 4th injection, the " flame " cells started to disintegrate releasing the amyloid staining material. These findings corroborate our previous results (Zlotnick and Tal, 1960).

The histological examination of the corresponding splenic biopsies from both groups of mice after the 6th injection showed diffuse and marked deposits of amyloid in the spleen. The intensity of the deposits varied among individual animals. The amyloid was usually present in both follicles and pulp with replacement of large areas of the parenchyma. At this stage plasma cells were rare or absent.

Serological studies.—The amyloid supernatant obtained from the spleens of mice after 6 injections of the saline-oil emulsion containing the TAB gave a positive Widal reaction and a low titre positive flocculation with the TB antigens. On the other hand, the ASF obtained from the spleens of mice injected with the saline-oil emulsion containing tubercle bacilli gave a high titre positive flocculation reaction with the tubercle bacillus antigen, but a negative Widal reaction.

The supernatant fluid prepared from normal mouse spleen (NSF) gave a negative Widal and a low titre TB flocculation reaction. At this stage, 7 weeks after the first injection, the pooled sera from the 2 groups reacted only with their respective antigens but the titre was somewhat lower than in the case of the ASF (Table I).

Tables II and III show the antibody titre of the serum and amyloid supernatant fluid at varying times after the injections of the respective antigens. At the 5th week (one week after the 4th injection) the sera showed no reaction at a

TABLE I.—*Agglutination of TAB and TB Antigens by Sera and ASF of Mice following Six Injections*

Material	TAB antigens						Flocculation with TB bacillus antigen					
	TO	TH	AO	AH	Bo	BH	1/10	1/20	1/40	1/80	1/160	1/320
TB injected mice:												
Serum	−	−	−	−	−	−	+	++	++	+	±	−
ASF	−	−	−	−	−	−	+++	+++	+++	+++	++	+
Typhoid injected mice:												
Serum	1/10 +	1/40 +	−	1/10 ±	−	1/20 ±	−	−	−	−	−	−
ASF	1/100 ++	1/200 ++	−	1/200 ±	1/100 ±	1/200 ++	++	++	+	+	±	−
Normal mice:												
Serum	−	−	−	−	−	−	−	−	−	−	−	−
NSF	−	−	−	−	−	−	+	+	±	±	−	−

ASF = amyloid supernatant fluid.
NSF = supernatant prepared from normal spleens.
− = no reaction.

stage at which the ASF already gave positive serological results. From the 6th week onwards antibodies appeared in the serum and increased in titre until the last examination performed at the 9th week (3 weeks after the last injection). Antibodies first appeared in the ASF after the 4th injection, at which stage most of the splenic smears showed amyloid-staining material. The titre subsequently increased, reached a maximum one week after the 6th injection, and 2 weeks later had decreased somewhat.

TABLE II.—*Agglutination of TAB Antigens by Sera and ASF at Different Intervals After Immunization*

Weeks	No. of injections	Antibody	TAB antigens					
			TO	TH	Ao	AH	Bo	BH
5	4	Serum	−	−	−	−	−	−
		ASF	1/100 ++	1/200 ±	−	1/100 ±	−	1/100 ±
6	5	Serum	1/10 ±	1/20 ±	−	−	−	−
		ASF	1/100 +	1/200 ±	−	1/100 ±	−	1/200 +
7	6	Serum	1/10 +	1/40 +	−	1/10 ±	−	1/20 ±
		ASF	1/100 ++	1/200 ++	−	1/200 ±	1/100 ±	1/200 ++
9	6	Serum	1/100 ++	1/200 ++	−	1/200 ±	1/100 ±	1/200 ++
		ASF	1/40 ±	1/200 +	−	1/100 ±	1/100 ±	1/200 +

Agar double diffusion test.—The rabbit TB–ASF antiserum gave 5 precipitation lines when tested against the homologous ASF, but only 4 precipitation lines when tested with the ASF prepared from the TAB injected mice (TAB–ASF) or with the supernatant fluid of normal mouse spleen (NSF). Similarly, rabbit

TABLE III.—*Flocculation of TB Bacillus Antigen by Sera and ASF at Different Intervals After Immunization*

Weeks	No. of injections	Antibody	TB bacillus antigens 1/10	1/20	1/40	1/80	1/160	1/320
5	4	Serum	—	—	—	—	—	—
		ASF	++	++	+	+	±	—
6	5	Serum	++	+	+	±	—	—
		ASF	+++	+++	+++	++	+	±
7	6	Serum	+	++	++	+	±	—
		ASF	+++	+++	+++	+++	++	+
9	6	Serum	+++	+++	+++	+++	++	+
		ASF	++	++	++	+	+	±

TAB–ASF antiserum gave 4 precipitation lines when tested against TAB–ASF, but only 3 lines against TB–ASF and NSF (Fig. 1).

After absorbing the 2 rabbit antisera with normal spleen, only one precipitation line remained with the homologous antigen (Fig. 2).

DISCUSSION

From the results obtained, it is evident that amyloid deposition in the spleen is preceded by a marked proliferation of plasma cells, which are transformed into " flame " cells which later disintegrate to liberate the amyloid staining substance. At the stage at which the amyloid material is deposited in the tissues, no or very few plasma cells can be found. These results are in complete accord with our previous findings (Tal and Laufer, 1960 ; Zlotnick and Tal, 1960).

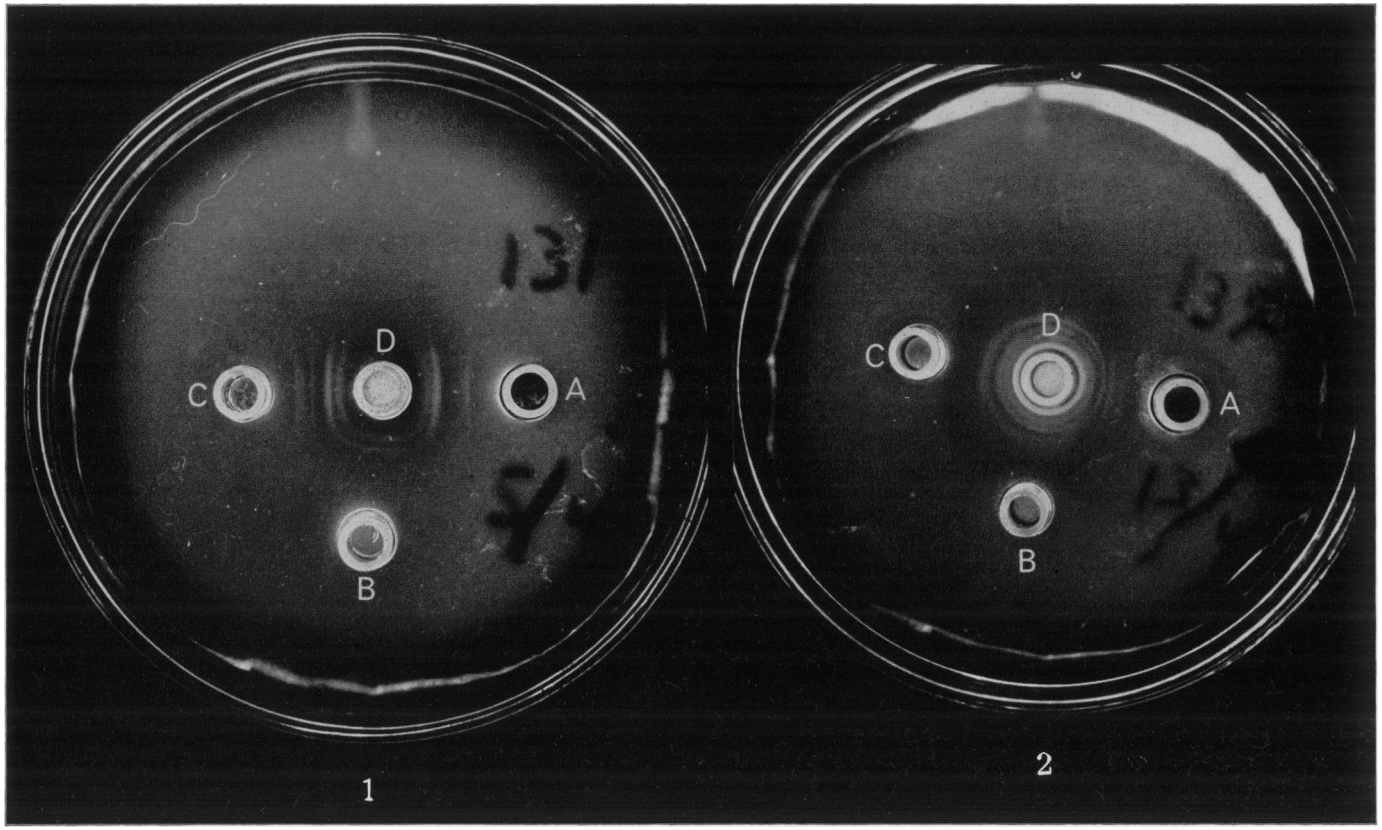
In previous publications by Letterer (1934) it was suggested that amyloid is produced by an immunologic reaction, and its deposition due to an antigen-antibody reaction. The presence of γ-globulin in the amyloid deposit in hyper-immunized animals observed with the aid of the fluorescence technique by Vasquez and Dixon (1956) lent further support to this hypothesis. Recently, Letterer has shown that these amyloid deposits contain complement fixing antigen antibody complexes (Letterer and Caesar, 1960).

We have attempted to show that the amyloid deposited in hyperimmunized mice possesses serological specificity to the antigen used in its production. The soluble crude amyloid prepared from the spleen of animals immunized with TAB gave a specific precipitation line only with its homologous antiserum. Similarly, the amyloid prepared from TB injected mice gave a positive precipitation line with its homologous antiserum only. Thus it seems evident that the amyloid, experimentally produced under the above conditions, is specific to the antigen to which it is produced. This does not necessarily imply, however, that amyloid is a pure antibody.

EXPLANATION OF PLATE

FIG. 1.—Agar double diffusion test. In the middle well D the TAB–ASF anti-serum ; note the 4 precipitation lines, tested against the TAB–ASF, well C, and the 3 precipitation lines tested against TB–ASF, well A, and against NSF (well B).

FIG. 2.—Agar double diffusion test. Absorption of anti-serum by NSF. The NSF is incorporated in the agar plate ; in the middle well D is the rabbit anti-TB–ASF serum ; in well A—TB–ASF ; in well B—NSF, and in well C—TAB–ASF. Note the single precipitation line tested against the homologous ASF.



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The agglutination and flocculation tests with the ASF became positive 2 weeks before positive results were obtained with the serum of the same animals. This might indicate that following cellular disintegration there is a release of this specific immunological reacting material into the serum. This is in accord with our morphological observation of disappearance of plasma cells at the stage at which amyloid has been deposited in the tissues (Tal and Laufer, 1960) and conforms with the electronmicroscopical studies of Letterer and Caesar (1960).

A possibility which had to be considered was that the immunological activity of the ASF was due to the presence of serum within the ASF preparations. This seems to be excluded by the fact that the ASF gave positive serological tests 2 weeks before antibody was demonstrable in the serum of the animals. This latter finding supports the view that the ASF itself contains the antibody reacting with the specific antigen.

The low titre cross-reaction between the normal splenic preparation (NSF) and tubercle bacillus antigen may be regarded as due to a shared common antigen between these two substances. Fortunately such cross-reaction was not encountered in the corresponding sera, so that the specificity of the TB flocculation reaction could still be appreciated.

SUMMARY

Amyloidosis was experimentally produced in mice by repeated injections of TB and TAB antigens in oil.

Studies of splenic smears and biopsies revealed that amyloid deposition was preceded by proliferation and subsequent disintegration and disappearance of plasma cells.

Serological studies have shown that the amyloid prepared from the spleens showed serological specificity to the antigen used for its production. At a later stage the sera of the injected animals also gave positive serological reactions with the corresponding antigen. The implication of this finding is discussed.

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