

# Amyloid-inducing factor and immunological unresponsiveness

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Several groups of investigators have shown that the induction time for experimental murine amyloidosis may be accelerated by passive transfer of viable spleen cells or subcellular fractions of spleen cells from casein-treated donors to isogenic recipients (Werdelin and Ranløv, 1966; Hultgren, Druet, and Janigan, 1967; Dreher and Letterer, 1970). On rare occasions the recipient mice appear to have developed amyloidosis without further treatment (Ranløv, 1967; Willerson, Gordon, Talal, and Barth, 1969), but in the majority of studies they have been subjected to additional manipulations including sublethal total body  $x$ -irradiation, administration of nitrogen mustard, or repeated injections of antigenic substances such as casein. For these and other reasons the mode of murine amyloid transfer has remained obscure and there has been virtually no agreement as to the nature of the cells or factors which are responsible for the accelerated induction of amyloid or even as to whether the pathogenesis of amyloid in the recipient animals is based on recognizable immunological principles. Indeed, Clerici, Mocarelli, De Ferrari, and Villa (1969) concluded that transfer amyloidosis was unlikely to be caused by adoptive immunity, since  $x$ -irradiated C57 inbred mice failed to develop amyloid after receiving intraperitoneal or intravenous injections of 5 to 30 million thoracic duct cells from isogenic amyloidotic donor animals, and Willerson and others (1969) produced evidence to suggest that an amyloid inducer or soluble amyloid rather than a specific functioning subcellular organelle was responsible for the transfer success in their experiments with Swiss white and C3H mice strains.

In the following paper we wish to report a series of experiments in which spleen and peritoneal cells were interchanged between casein-treated and non-casein-treated outbred guinea-pigs. In these experiments, guinea-pigs receiving intraperitoneal injections of spleen cells from donors rendered tolerant to casein developed amyloid at an accelerated rate. This

finding suggests the presence of a subcellular factor which is capable of crossing histocompatibility barriers and which may be a mediator of high-dosage immune paralysis as well as amyloidosis.

## Material and methods

### DONORS

Sixteen white outbred Hartley guinea-pigs were started on an amyloid-inducing regimen consisting of thrice weekly 1 ml. subcutaneous injections of 12 per cent. sodium caseinate (Mann Research Inc., New York); 5 weeks after the first casein injection these guinea-pigs were immunized by foot-pad injections of an emulsion containing diphtheria toxoid (Dtd—total dose 17 flocculating (Lf) units) and horseradish peroxidase (HRP—total dose 0.4 mg.) in complete Freund's adjuvant, and 3 weeks later four of these animals were tested for cellular immune responsiveness to casein, Dtd, and HRP according to macrophage inhibition tests as previously described (Cathcart, Mullarkey, and Cohen, 1971). The twelve remaining guinea-pigs (that had also been immunized to Dtd and HRP) each received an intraperitoneal injection of 20 ml. light mineral oil, and 3 days later their peritoneal cells and spleen cells were harvested for subsequent transfer to non-casein-treated recipient guinea-pigs. Before the latter procedure blood was taken from the portal veins of all the donor animals and tested for precipitating antibodies to casein, Dtd, and HRP by double diffusion studies in agar (Ouchterlony, 1958, 1962).

Small pieces of the donor spleens were removed for histological examination and the remainder was placed in Hanks's balanced salt solution (Microbiological Associates, Bethesda, Maryland), gently teased apart, homogenized by hand in a Potter Elvehjem apparatus at 0°C., and passed through a gauze filter. The resultant pooled suspension of cells was washed and centrifuged three times to provide sensitized donor cells that were 90 to 95 per cent. viable as tested by exclusion of trypan blue. Peritoneal cells were also washed and centrifuged three times in Hanks's solution and the final pooled suspension consisted of 80 to 85 per cent. macrophages and 15 to 20 per cent. small lymphocytes and polymorphonucleocytes.

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A second series of donor spleen cells and peritoneal cells (*i.e.* non-sensitized donor cells) was similarly prepared from twelve control Hartley guinea-pigs that neither received casein injections nor were immunized against Dtd and HRP.

#### RECIPIENTS

In the transfer experiments 52 recipient Hartley guinea-pigs of the same outbred strain were injected intraperitoneally with either 200 million spleen or peritoneal cells from casein-treated or non-casein-treated donors. After 1 week, four of the guinea-pigs receiving spleen cells from sensitized donors were subjected to the macrophage inhibition test and their serum was examined for precipitating antibodies to casein, Dtd, and HRP. These animals were then killed and portions of their spleens, livers, and kidneys were examined histologically for the presence of amyloid. Seven days after the initial transfer of cells all the remaining recipients, comprising 48 animals, were subdivided into four subgroups according to whether they had been given sensitized or non-sensitized spleen cells or macrophages. These subgroups comprising twelve animals each were further divided so that half of the animals were started on thrice weekly casein injections and the other half were left untreated. All recipient guinea-pigs were killed in pairs at periods varying from one to 5 weeks after transfer.

#### CONTROLS

Eight guinea-pigs were started on thrice weekly casein injections at the same time as the donor guinea-pigs. These animals were killed in groups of four 10 and 12 weeks after the institution of casein injections in order to study the rate of development of amyloidosis in casein-treated guinea-pigs.

#### MACROPHAGE INHIBITION TEST (MIT)

Three days before testing, guinea-pigs were injected intraperitoneally with 20 ml. light mineral oil (Fisher Scientific Company, Medford, Massachusetts). After the guinea-pigs had been bled by cardiac puncture, peritoneal cells were harvested using sterile techniques, washed in Hanks's solution, packed into capillary tubes, and placed in tissue culture chambers (two tubes/chamber). The chambers were filled with minimal essential medium (Microbiological Associates, Bethesda, Maryland) containing 15 per cent. normal guinea-pig serum, 1 per cent. L-glutamine, 1 per cent. penicillin, 1 per cent. streptomycin, and either casein (0.12 mg.), Dtd (36 Lf units), Mv (0.06 ml.), or HRP (0.1 mg.). The tubes were then sealed and incubated for 24 hrs at 37°C. The resultant cell images were projected and traced, and the areas of migration were measured by planimetry. Areas of migration in duplicate test chambers were averaged and the results were expressed as the percentage of migration of peritoneal cells in the control chambers containing no antigens. Positive inhibition in these experiments was recorded when macrophage migration was less than 85 per cent. of macrophage migrations in the controls ( $P < 0.01$ ).

#### HISTOLOGICAL STUDIES

Kidney, liver, and spleen sections of each test animal were fixed in formalin, sectioned, and stained with Congo red. The presence of amyloid was ascertained by viewing each section under polarized light for green birefringence.

## Results

#### IMMUNOLOGICAL STUDIES

All four guinea-pigs receiving thrice weekly subcutaneous injections of 12 per cent. casein for 8 weeks demonstrated negative MITs to casein (96 per cent. mean macrophage migration) and positive MITs to Dtd and HRP (Dtd, 75 per cent. mean macrophage migration; HRP, 50 per cent. mean macrophage migration). None of the four recipient guinea-pigs demonstrated a positive MIT to casein, Dtd, or HRP when tested 7 days after the transfer of sensitized spleen cells (Table I).

**Table I** *Results of macrophage inhibition tests in donor and recipient guinea-pigs*

Immunogen	Macrophage migration (per cent.)*	
	Donor	Recipient
Dtd	75	116
HRP	50	116
Casein	96	108

\* Positive inhibition when macrophage migration is less than 85 per cent. migration in controls ( $P < 0.01$ )

Sera from all twelve donor guinea-pigs produced a single line of precipitation when tested by double immunodiffusion against Dtd and HRP. Precipitating antibodies to casein were not detectable in these animals. None of the sera from the four recipient guinea-pigs had detectable antibodies to casein, Dtd, or HRP using the same techniques.

#### HISTOLOGICAL STUDIES

Amyloid was found in all six guinea-pigs that received sensitized spleen cells and were subsequently started on thrice weekly casein injections (Table II, Group A, overleaf).

Amyloid deposits were most noticeable in the perifollicular zones of the spleen; they were not observed in the liver or kidney. Amyloid deposits in animals receiving casein injections for 2 and 5 weeks were more noticeable and stained more intensely with Congo red than those observed in animals killed only 1 week after the first casein injection (Figure). Amyloid was also present to a lesser degree in the spleens of animals given sensitized peritoneal cells and casein (Table II, Group B) and then not until the 4th week after transfer and 3 weeks after the first casein injection.

None of the animals given sensitized spleen cells and no casein, or sensitized peritoneal cells and no casein (Table II, Groups C and D) developed amyloidosis when transfer was carried out 1, 2, 3, and 4 weeks previously. In addition, none of the guinea-pigs that received non-sensitized spleen or peritoneal cells (Table II, Groups E, F, G, and H) had demonstrable amyloid in their tissues when killed 3, 4, and

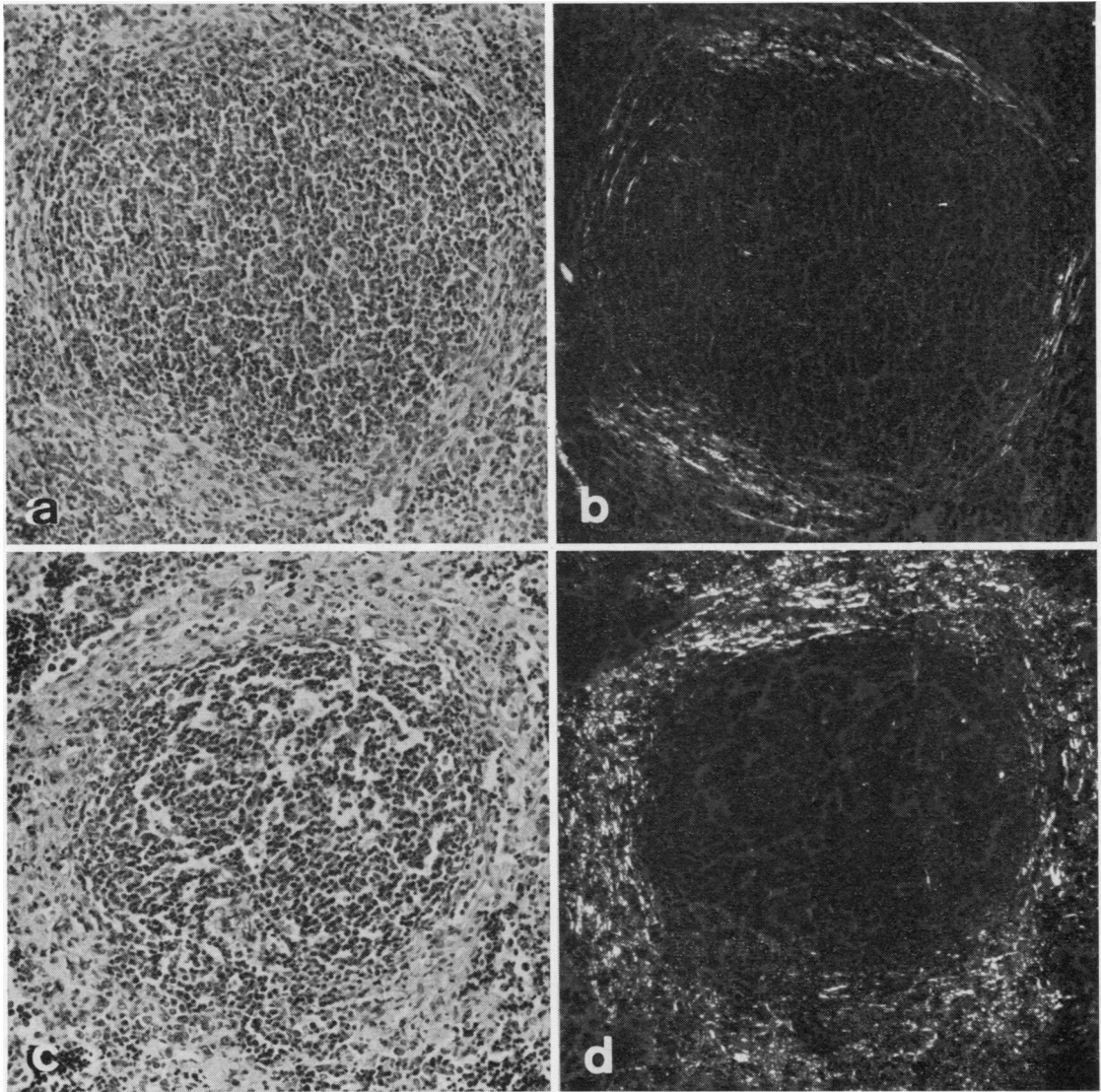


FIGURE Splenic amyloid as observed by light microscopy (a) and by polarization microscopy (b) after 1 week of casein injections, and as observed by light microscopy (c) and by polarization microscopy (d) after 3 weeks of casein injections. Congo red stain.  $\times 140$

5 weeks after transfer, whether they had received multiple casein injections or not.

Amyloid was not found in the tissues of donor animals that received thrice weekly injections of casein for 8 weeks. On the other hand amyloid was detected in trace amounts in two of four animals receiving casein for 10 weeks and was present in all of the spleens of guinea-pigs killed after 12 weeks of casein injections.

#### Discussion

These data indicate for the first time that a species

other than the mouse can be used for the study of transfer amyloidosis. Although the number of animals in each experimental group was not large it was considered highly significant that all six guinea-pig recipients in Group A developed amyloidosis. Thus, it was possible to decrease splenic amyloid formation from the customary 12-week induction period to less than 1 week after the first casein injection. As previously observed in mice (Janigan and Druet, 1968; Shirahama, Lawless, and Cohen, 1969), the accelerated induction of amyloid in guinea-pigs was found to be dependent on a factor which is capable of crossing histocompatibility bar-

**Table II** *Amyloid in guinea-pigs receiving spleen cells and peritoneal cells from sensitized and non-sensitized donors*

Groups of guinea-pigs		No. of weeks after transfer				
		1	2	3	4	5
Receiving sensitized cells	A Spleen cells and casein		+	+	+	
	B Peritoneal cells and casein		-	-	+	
	C Spleen cells	-	-	-	-	
	D Peritoneal cells		-	-	-	
Receiving non-sensitized cells	E Spleen cells and casein			-	-	-
	F Peritoneal cells and casein			-	-	-
	G Spleen cells			-	-	-
	H Peritoneal cells			+	-	-

+ = definite green birefringence by polarization microscopy.  
 - = negative for amyloid.

riers. In the latter respect, the amyloid-inducing factor seems to differ from several mediators involved in cellular immunity, since the macrophage inhibition factors to Dtd and HRP were lost within 7 days after the intraperitoneal injections of sensitized spleen cells into recipient animals (despite positive transfer of amyloid-inducing factor). This finding is in keeping with previous reports that delayed hypersensitivity, as measured by positive skin tests, is rapidly lost when transfer is carried out between outbred guinea-pigs (Chase, 1969).

Recent studies from our laboratory have suggested that the specific pathogenic role of casein in experimental amyloidosis may be that of a tolerogen, rather than an antigen as was previously thought (Cathcart, Mullarkey, and Cohen, 1970). This hypothesis was based on the finding that guinea-pigs receiving thrice weekly subcutaneous injections of casein at first developed a cellular immune response to casein but that this rapidly disappeared after 3 or more weeks of casein administration (Cathcart and others, 1971; Briccetti, Cathcart, and Cohen, 1971). The results of the present study both confirm and extend these observations, since it was possible to show that all donor pre-amyloidotic guinea-pigs had detectable circulating antibodies and positive macrophage inhibition tests to Dtd and HRP respectively at a time when they appeared to be unresponsive to casein using the same techniques. Indeed, one of the most interesting features of the present study was the discovery that the accelerated induction of amyloid in recipient guinea-pigs also appeared to be dependent on the presence of the inducing agent casein, *i.e.* only those recipients given repeated casein injections developed amyloid in 1 to 3 weeks. This finding suggests that tolerance to specific antigens may also play a significant pathogenic role in the transfer model of amyloidosis and has prompted a series of additional studies in which recipient guinea-pigs will undergo direct testing of tolerance to casein and non-specific antigens.

Although the mechanisms involved in high-dosage tolerance or immune paralysis are not fully understood, it has recently been postulated that they may be dependent upon cooperation or interplay between thymus-dependent lymphocytes and bone marrow cells. It may be that changes in cell membranes induced by tolerogenic antigens are different from those triggered by immunogens, and it has recently been proposed that loss of immunological responsiveness may be initiated by an event such as the shedding of antigenic receptor sites from immunocompetent cells (Smith, 1969). The results obtained in the present study further support the notion that the pathogenesis of immune paralysis and amyloid may be intimately related and suggest that both phenomena may be mediated or enhanced by a factor similar to that demonstrated in the guinea-pig transfer model.

### Summary

Using a single intraperitoneal injection of pre-amyloidotic spleen cells or peritoneal cells, it was possible to produce accelerated amyloid formation in recipients from an outbred guinea-pig strain. Results of immunological studies carried out in the donor animals were consistent with the notion that the pathogenesis of experimental amyloidosis may be related to the induction of high-dosage tolerance to the inducing agent casein. Results of immunological studies performed in the recipient animals indicated that the pathogenesis of transfer amyloidosis in guinea-pigs may be due to a factor which is independent of the various mediators involved in cellular immunity but is dependent on chronic casein stimulation.

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### Discussion

DR. W. W. BUCHANAN (*Glasgow*) Are there any animals which do not develop amyloid when challenged with casein and if so what is the immune status of these animals?

DR. CATHCART All the animals that were tested by us developed amyloidosis. However, it is noteworthy that rats given casein for many months develop circulating antibodies to casein but fail to develop amyloidosis.

PROF. E. G. L. BYWATERS (*Taplow*) Ranløv (1967) claimed that he could obtain transference with cell-free serum fluid. Do we know more about this? Could this transfer factor be extra-cellular?

Has rosette formation been studied to indicate antigen recognizing lymphocytes?

DR. CATHCART From many studies in mice it is clear that a subcellular or extracellular factor is responsible for the transfer between syngeneic animals and our studies agree. Our guinea-pig cells appear to have been destroyed after transfer across histocompatibility barriers yet the factor still operates. Willerson and others (1969), at the National Institutes of Health, have shown that several subcellular fractions—microsomal, mitochondrial, and nuclear—are equally involved, suggesting that an amyloid-inducer or soluble amyloid rather than a specific functioning organelle is responsible for the transfer success.

Finally, I have discussed tolerance but have not mentioned the humoral arc. Several investigators claim that casein antibodies may be present in experimental amyloidosis and if so then this is not strictly tolerance but "split" immunological paralysis. Rosette formation, plaque techniques, etc., have not been investigated but these studies are in progress in our laboratory.

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