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Cellular Immunity in Casein-Induced Amyloidosis

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Summary. Guinea-pigs receiving multiple subcutaneous injections of casein at first developed a cellular immune response to casein but this rapidly waned when the animals became amyloidotic. By contrast, cellular immune responsiveness to non-specific antigens at first appeared to be diminished but later returned to normal under the same conditions. These results suggest that tolerance to a specific immunogen may play an important role in the pathogenesis of casein-induced amyloidosis.

INTRODUCTION

Although casein is frequently used to induce amyloidosis in animals, the immunological properties of this protein and its relation to the disease induced are largely unknown. After the discovery by Vasquez *et al.* that specific antigen and antibodies were present in casein-induced amyloid deposits in rabbits (Vasquez, Dixon and Neil, 1957), it appeared possible that amyloid might be formed by direct tissue precipitation of immune complexes. Nevertheless, the pathogenic role of antibodies to casein seemed less important when Clerici, Pierpaoli and Romussi (1965) showed that rabbits, immunologically paralysed to casein while neonates, developed, when mature, as much amyloid as their litter mates in response to a prolonged course of casein injections. On the other hand, Letterer and Kretschmer (1966) described a similar study in which the incidence of experimental amyloidosis was reduced following the development of tolerance in neonatal mice. However, no proof of the unresponsiveness was provided in the latter report.

Even less information is available concerning the effects of chronic casein administration on the cellular aspects of the immune response. Ranlov and Jensen (1966) were first to show delayed homograft rejection in mice rendered amyloidotic by multiple injections of subcutaneous casein and Rodey, Becker and Pisciotta (1968) found that a single injection of azocasein in the mouse suppressed haemagglutinin and haemolysin responsiveness to sheep red cells but did not affect the agglutinin response to brucella antigens in the same animal. They concluded that these findings parallel the effects of thymectomy on the humoral response in the same species. More recently, it has been shown that the *in vitro* response to phytohaemagglutin by mouse spleen cells is diminished by short-term injections of casein, azocasein or Freund's complete adjuvant. Animals in these experiments, however, did not appear to develop amyloidosis and it was also noted that casein produced non-specific mitogenic activity when incubated with normal cells *in vitro* (Rodey and Good, 1969).

In the present investigation we have attempted to examine the cellular immune response to casein using the *in vitro* macrophage test (David, Al-Askari, Lawrence and

Thomas, 1964). Casein was tested, first for its effect solely as an antigen, and second, for its antigenic properties while being used as an amyloidotic agent. Finally, we attempted to determine the effects of chronic casein administration on cellular immune responses to a variety of non-specific antigens including diphtheria toxoid (Dtd), mumps vaccine (Mv) and horse-radish peroxidase (HRP). The methods used to perform these studies and the results obtained are as follows.

EXPERIMENTAL METHODS

Animals

Guinea-pigs of the Hartley strain, weighing 350–500 g were used.

Antigens

For footpad and multiple subcutaneous injections, casein was prepared by dissolving sodium caseinate (Mann Research Inc., New York) in boiling water in a Waring blender for 1 minute at a concentration of 12 g/100 ml. For use as an antigen in the macrophage inhibition test, aliquots were dialysed overnight against phosphate buffered saline, 0·01 M, pH 7·1. Horse-radish peroxidase, type II (HRP) was obtained from Sigma (St Louis, Missouri) diphtheria toxoid (Dtd) was obtained from the Commonwealth of Massachusetts, Department of Public Health, Division of Biological Laboratories and mumps vaccine (Mv) was obtained by courtesy of Eli Lilly and Company (Indianapolis, Indiana).

Immunization and sensitization

Guinea-pigs were immunized with 1 ml of 12 per cent casein, three times per week, for periods varying between 0 and 52 weeks. At specified intervals, groups of three guinea-pigs were injected with casein, (total dose 8·2 mg), Dtd (total dose 17 flocculating (Lf) units), Mv (total dose 0·17 ml) and HRP (total dose 0·4 mg) by injecting each of four footpads with an emulsion containing one or more of the antigens in Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan).

Macrophage Inhibition Test (MIT)

Three days before testing, guinea-pigs were injected intraperitoneally with 20 ml of light mineral oil (Fisher Scientific Company, Medford, Massachusetts). After the guinea-pigs were exsanguinated 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 chambers were then sealed and incubated for 24 hours at 37°. The resultant cell images were projected, traced, and the areas of migration measured by planimetry. Areas of migration in duplicate test chambers were averaged and the results expressed as the per cent 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 65 per cent of macrophage migration in the controls ($P \leqslant 0{\cdot}01$).

Histological Studies

Following each experiment, kidney, liver and spleen sections of each test animal were fixed in formalin, sectioned and stained with Congo red. The presence of amyloid in the various tissues was determined by viewing each section by polarization microscopy for green birefringence.

RESULTS

Experiment I

In the first experiment the immunogenicity of casein and Dtd was tested by sensitizing a group of eighteen guinea-pigs at week 0 and then performing MITs at weeks 1, 2, 3, 4 and 7, respectively. Significant macrophage inhibition to both antigens was demonstrable by the end of the first week (casein, 38 per cent mean macrophage migration; Dtd, 57 per cent mean macrophage migration) and persisted through weeks 2, 3, 4 and 7 (Fig. 1,

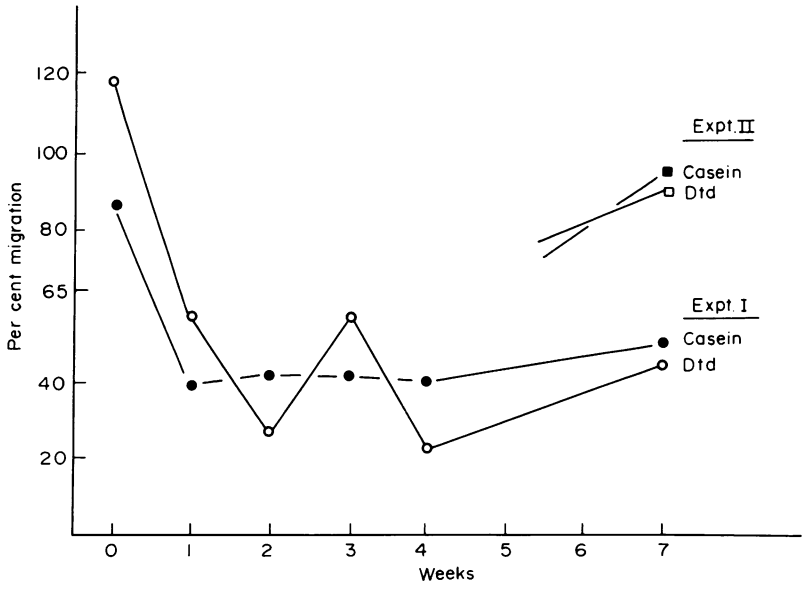

Fig. 1. Macrophage inhibition tests performed in guinea-pigs sensitized with casein and Dtd (Exp. I). At week 7, MITs were also performed on animals receiving multiple casein immunizations for 4 weeks (Exp. II).

Exp. I). Control experiments revealed that macrophages from non-sensitized animals were not inhibited by casein or Dtd (casein, 87 per cent mean macrophage migration; Dtd, 118 per cent mean macrophage migration).

Experiment II

From the same group of animals that were used in Experiment I, three were selected at the end of week 3 and given tri-weekly subcutaneous casein immunizations for 4 weeks. MITs were performed at week 7 in order to determine if numerous casein immunizations

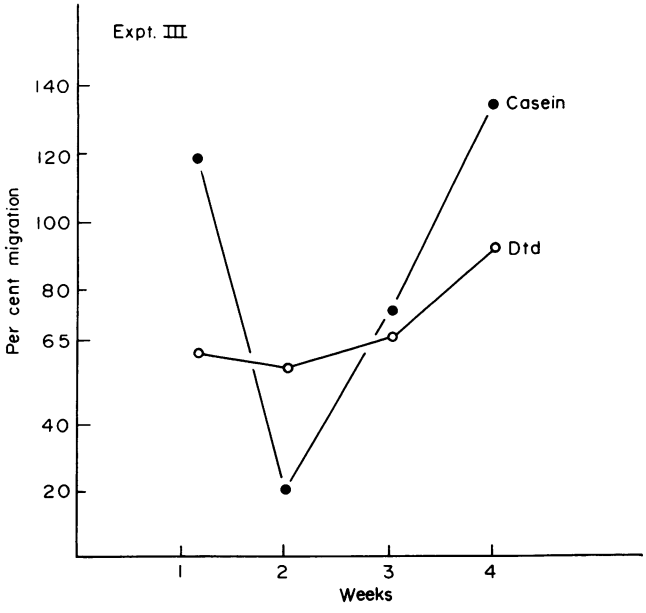

FIG. 2. Macrophage inhibition tests performed at weekly intervals in guinea-pigs receiving multiple casein immunizations (Exp. III). This group of animals was sensitized to Dtd at the commencement of the multiple casein injections.

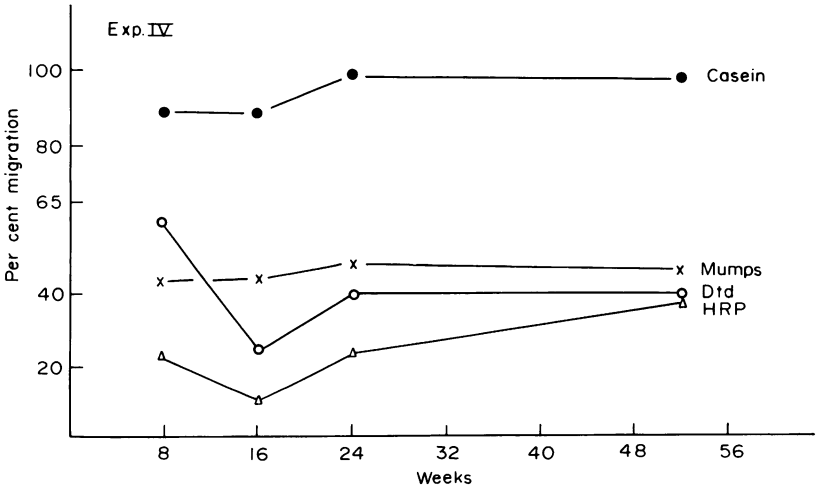

FIG. 3. Macrophage inhibition tests performed in guinea-pigs 8, 16, 24 and 52 weeks following multiple casein immunizations (Exp. IV).

altered the immune responsiveness of animals already sensitized to casein and Dtd. It was found that each of the animals developed unresponsiveness to both casein (94 per cent mean macrophage migration) and Dtd (93 per cent mean macrophage migration) at week 7 (Fig. 1, Experiment II).

Experiment III

A second group of animals, comprising twelve guinea-pigs, were started on tri-weekly casein immunizations, but at the same time were sensitized with footpad injections of Dtd in Freund's complete adjuvant. By week 2 significant macrophage inhibition to both

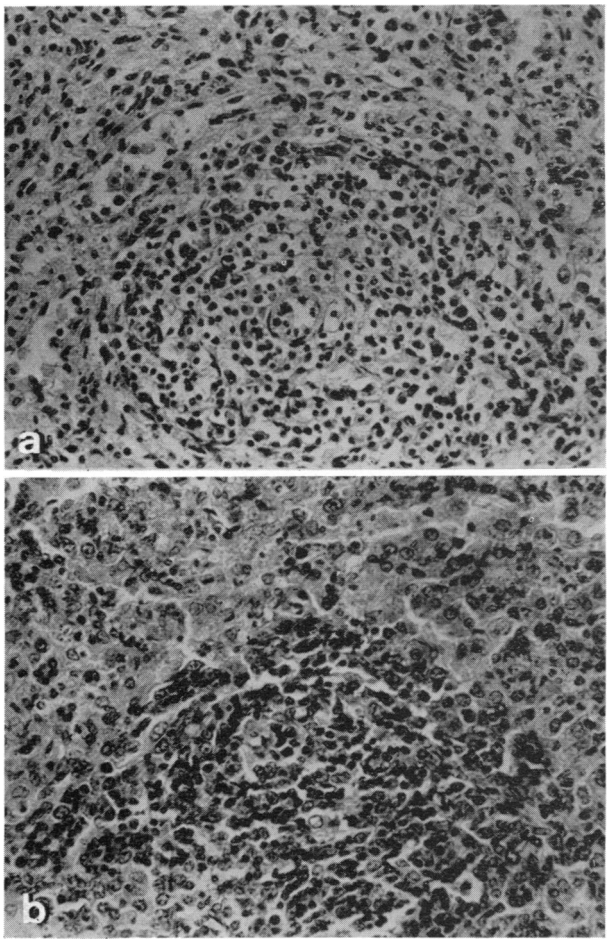

Fig. 4. (a) Congo red stain of a guinea-pig spleen 2 weeks after the onset of multiple casein immunizations. There is hypercellularity in both the white and red pulp. × 128. (b) Congo red stain of guinea-pig spleen 8 weeks after the onset of multiple casein immunizations. There is marked cellular infiltration of macrophages and reticulum cells in the perifollicular zone. × 128.

casein (20 per cent mean macrophage migration) and Dtd (57 per cent mean macrophage migration) had developed. However, at week 3, sensitivity to both antigens appeared to decline (casein, 74 per cent mean macrophage migration; Dtd, 66 per cent mean macrophage migration) and by week 4 delayed hypersensitivity to both antigens was almost completely abolished (Fig. 2).

Experiment IV

In the final series of experiments, twelve guinea-pigs were placed on tri-weekly casein immunizations. Groups of three animals were subsequently sensitized to Dtd, Mv and HRP at weeks 5, 13, 21 and 49, respectively. MITs to the specific amyloidogenic agent,

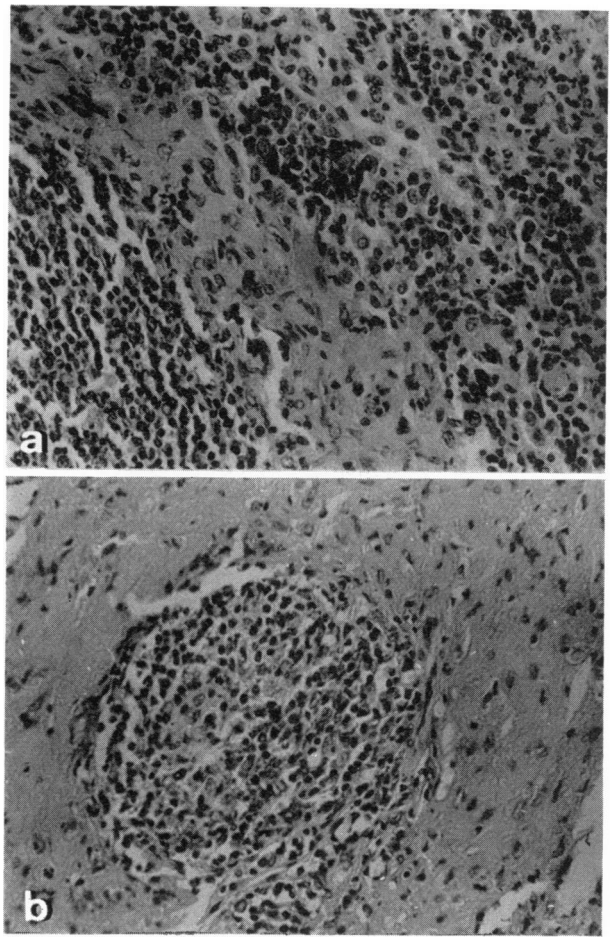

Fig. 5. (a) Congo red stain of a guinea-pig spleen at week 12. There is abundant newly formed amyloid in the perifollicular zone. × 128. (b) Congo red stain of guinea-pig spleen 52 weeks after the onset of multiple casein immunizations. There is almost total replacement of the red pulp by amyloid. The relative hyperplasia of the lymphoid follicles is probably related to recent antigenic challenge with Dtd, Mv and HRP. × 128.

casein, and the three non-specific antigens, Dtd, Mv and HRP were then carried out at weeks 8, 16, 24, and 52. It was found that unresponsiveness to casein persisted throughout the 52-week period whereas significant macrophage inhibition to each of the non-specific antigens was present at weeks 8, 16, 24 and 52 (Fig. 3). Control experiments confirmed that macrophages from non-sensitized animals were not inhibited by casein, Dtd, Mv or HRP.

APPEARANCE OF AMYLOIDOSIS IN GUINEA-PIG TISSUES

Two weeks after subcutaneous casein injections hyperplasia in both the white and red pulps of the spleen was apparent (Fig. 4a). By week 8 the perifollicular zones of the spleen demonstrated a marked cellular infiltration of macrophages and reticulum cells (Fig. 4b). By week 12 the earliest deposits of amyloid were observed in the perifollicular areas (Fig. 5a) and by week fifty-two large splenic deposits of amyloid were present in all spleens tested (Fig. 5b). In the later stages of the study additional deposits of amyloid were seen in the peritubular areas of the kidneys and the sinusoidal areas of the liver. Amyloid was not found in animals that did not receive multiple injections of casein and were sensitized by footpad injections, only.

DISCUSSION

The results of our initial experiments clearly showed that casein can be used as an immunogen to produce delayed hypersensitivity in guinea-pigs as measured by the macrophage inhibition technique. Responsiveness to this immunogen is achieved not only following its administration in Freund's complete adjuvant but also shortly after the institution of multiple subcutaneous injections. Control experiments also ascertained that casein as well as the other non-specific antigens that were used throughout these studies do not interfere with cell migration of normal or unsensitized macrophages.

In subsequent studies it was noted that the institution of subcutaneous injections interfered with cellular responsiveness to a second non-specific antigen, Dtd, when the latter was administered at the same time as the first casein injection. These findings of non-specific inhibition of delayed hypersensitivity recall those obtained by Liacopoulos and Neveu (1964), who discovered non-specific tolerance to a second antigen when either bovine serum albumin or horse γ-globulin was used as the principal immunogen. Although the precise mechanisms governing this phenomenon are not known it has been postulated that the impaired responsiveness to the second antigen is due to antigenic competition (Adler, 1957; Asherson, 1967). This explanation may not suffice for the present findings, however, since it could be demonstrated that sensitization and memory for the non-specific antigen Dtd was well established prior to the onset of multiple casein immunizations. Indeed, these results suggest that *in vitro* unresponsiveness to Dtd at week 7 was probably due to competition by cells already committed to casein recognition. Furthermore, they bolster the notion that immunological tolerance to a specific antigen is a positive expression of cellular recognition (Dresser and Mitchison, 1968) in that those lymphocytes which were tolerant to casein were capable of interfering with the *in vitro* macrophage response to Dtd.

Our last series of experiments showed that unresponsiveness to casein which develops in guinea-pigs within 3 weeks and persists for 1 year does not interfere with the ability of these same animals to respond to three other antigens, one of bacterial origin, i.e. diphtheria toxoid, one of viral origin, i.e. mumps vaccine and one with specific enzymatic activity, i.e. horse-radish peroxidase. Thus, a relatively normal cellular immune function was demonstrated despite observations by others that thymectomy, splenectomy, ionizing radiation, antimetabolites, cortisone, parabiosis, and other conditions which favour the depletion of small lymphocytes all appear to enhance the development of amyloid in various species (Muckle, 1968). It is noteworthy, however, that Lehner, Cameron and

Ward (1970) recently reported that lymphocytes from patients with amyloidosis were capable of normal blast formation when stimulated with phytohaemagglutinin and a variety of non-specific antigens. It remains to be shown whether comparable results will be obtained in amyloidotic guinea-pigs when similar techniques are employed using specific and non-specific antigens.

In conclusion, the cellular immune response to casein and a variety of non-specific immunogens has been measured for the first time in amyloidotic and non-amyloidotic guinea-pigs. It has been shown that specific cellular unresponsiveness to casein develops during the induction of amyloidosis while other functions of cellular immunity remain intact. Previously conflicting data on the role of the immune response in the genesis of amyloid might be reconciled if the theory is borne out that tolerance to a specific immunogen plays a central role in this disease (Cathcart, Mullarkey and Cohen, 1970).

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