Experimental Murine Amyloidosis in X-Irradiated Recipients of Spleen Homogenates or Serum from Sensitized Donors

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It has been shown recently that experimental amyloidogenesis in mice can, at some stage, involve the participation of lymphoreticular cells from sensitized donors. Lethally X-irradiated C57BL/10J mice were given isogeneic spleen cells from untreated donors or from donors previously sensitized to azocasein. The same antigen was then injected briefly into all recipients. Only those recipients of cells from sensitized donors developed amyloidosis. Amyloid fibers were spatially related to cells believed to be fixed reticuloendothelial (RE) cells of the recipient; the possibility that the latter were donor cells that both colonized the recipient and elaborated amyloid could not, however, be discounted. The results of the present studies, which were presented in part elsewhere, exclude this possibility.

Materials and Methods

Inbred C3H/HeJ and C57BL/10J male mice, 6–8 weeks old, were used. Azocasein was synthesized as reported earlier. A 10% solution (w/v) in 0.01 M NaHCO₃ was prepared for injection. Total body X-irradiation with 400 or 750 rads was done under the following conditions: 300 kv, 20 ma, HVL 2-mm. copper filter, 50 cm. FSD to give a mean air dose of 177 rads/min.

Donor Spleen Cell and Spleen Homogenate Preparations

Donor mice were exsanguinated during light ether anesthesia. Serum was collected and stored at −20° C. for up to 3 months. Spleens were immediately weighed, pooled, and diced in ice-cold Hanks' fluid. Intact spleen cells were isolated as described previously. Homogenates were prepared by extensively grinding spleens (20 passes with a motor-driven pestle) in Hanks's fluid with a tight-fitting Potter-Elvejheim homogenizer in an ice bath. The homogenates were filtered through a 150-µ mesh wire screen, and then frozen (−76° C.) and thawed (37° C.) twice. As seen by microscopy, no intact, nucleated cells were found in random homogenate samples. All homogenates were used immediately, except in one instance when the homogenate was stored at −20° C. for 3 weeks.

Procedural Details

Certain procedures were common to much of the experimental design outlined in the next section. The antigen or its solvent, NaHCO₃, was injected subcutaneously in 0.3-ml volumes on consecutive days. All mice, whether donor or recipient, were killed 24 hr. after the last antigen injection.

All donor materials were transferred intraperitoneally into recipients which had been X-irradiated 2–4 hr. previously. After 24 hr. the recipients were started on injections of azocasein. The spleen cell or homogenate transfers were calculated to deliver the equivalent of one donor spleen to each recipient. Since the spleens from sensitized donors weighed as much as 3 times more than those from nonsensitized donors, as many as 3 spleens from the latter were transferred to each recipient to maintain equivalence. Pooled donor serums were injected into recipients, in arbitrary 0.4-ml amounts.

With the few exceptions noted later, all mice were necropsied. Blocks of multiple organs were fixed and processed for paraffin sections as described previously. Selected blocks of spleen and liver were fixed and embedded for electron microscopy. All paraffin sections were routinely stained with Congo red. The histologic diagnosis of amyloid was based partly on morphologic features and, more stringently, on both the binding of Congo red and the properties of green birefringence and dichroism in polarized light. Selected specimens were stained with the indole method for tryptophan, or examined with the electron microscope to confirm the diagnosis of amyloidosis.

Experimental Design and Results

To facilitate descriptions of the results, the design of the tests will be given in this section with text-figures. Irrespective of the conditions of induction, the morphologic, tinctorial, and ultrastructural characteristics of the amyloid fibers were constant, and similar to those previously described. For present purposes, the incidence of amyloidosis will be based on the finding of amyloid in the spleen, since in its absence no other organs were involved. Other details will be reported separately.

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Text-fig. 1. Groups A, B, and C (intact C₅H mice) which received injections of azocasein for 4, 7, and 9 consecutive days and were killed 1 day later. Mice in group D previously received injections of azocasein (A-C) for 7 days (D). After 1-day interval, injections were resumed for 2 or 4 consecutive days. Numbers (No.) of mice used and found to have amyloidosis are indicated for each group.
Azocasein (Text-fig. 1)

Azocasein was injected into intact and previously untreated C3H mice for 4, 7, and 9 days (Groups A, B, and C, respectively). Amyloidosis was found only after 9 injections (Group C). Another group (Group D) received injections for 7 days. After a 1-day interval during which no injections were made, these mice were again given injections of azocasein for 2 or 4 days. All mice developed amyloidosis.

These results simply show that, with the batch of antigen used, a certain number of daily injections—i.e., 9—was sufficient for amyloid induction and that a smaller number—i.e., 4 or 7—was inadequate. The requirement for at least 9 injections is not changed by interspersing a nontreatment day in the sequence.

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![Diagram](image)

Text-fig. 2. Top, intact C3H mice given total body X-irradiation. Mice in Group C were X-irradiated (X-IRRAD.) and 24 hr. later were given injections of azocasein for 4 days. Bottom, intact C3H mice (donors) were given injections of NaHCO₃ for 7 days. Donor spleens or serum were transferred to previously X-irradiated recipients (Groups D and E) and 24 hr. later, the recipients received injections of azocasein (TREATMENT) for 4 days (A-C x 4D).

X-irradiation and Azocasein (Text-fig. 2)

Intact and previously untreated C3H mice were X-irradiated with 750 or 400 rads (Groups A and B, respectively). All mice in Group A died Feb. 1968
within 13 days; half the mice of Group B were dead by 19 days. Gross and/or histologic necropsy examination of nearly half the mice dying after both X-ray dosages revealed no amyloidosis. No amyloid was found in mice (Group C) which were X-irradiated (9 with 750 rads, 5 with 400 rads), and then given 4 azocasein injections.

Donor mice were given injections of sodium bicarbonate for 7 days. Their spleens, in the form of either intact cells or homogenates, were transferred into irradiated, isogeneic recipients, (Group D, consisting of equal numbers of mice exposed to 400 or 750 rads). The recipients were then injected with azocasein for 4 days. Two mice died after 2 injections. Amyloidosis was not found in any mice, or in similarly treated X-irradiated recipients (Group E, 750 rads) of serum from the same donors.

The results (Text-fig. 2) demonstrate, therefore, the failure to induce amyloidosis after 4 azocasein injections in either lethally or sublethally X-irradiated mice, or in irradiated recipients of spleen tissue or serum from nonsensitized donors.

**Azocasein for 7 Days (Text-fig. 3 and 4)**

The tests demonstrated in Text-fig. 3 were identical to those depicted in the lower part of Text-fig. 2 with the exception that the donor mice received injections of azocasein for 7 days. Splenic biopsies or necropsy of the donors revealed no amyloidosis (see also Text-fig. 1, Group B).

The recipients of Group A consisted of 55 and 24 mice exposed to 750 and 400 rads, respectively; they received either intact spleen cells or...
spleen homogenates from the sensitized donors. Nearly all developed extensive amyloidosis after 4 azocasein injections. There were no significant differences in the amyloidosis of mice given either radiation dosage in combination with either spleen preparation. Storage of spleen homogenates at \(-20^\circ\) C. for 3 weeks did not affect the uniformity of these results. Two recipient mice died after 2 injections of azocasein but were not examined. Of another 4 mice dying after 3 injections, 3 showed slight amyloidosis.

The mice in Group B, all given 750 rads, received injections of serum from the sensitized donors and, as above, received 4 azocasein injections. Two mice died after 2 injections. Of the remaining 7 mice, 6 showed amyloidosis which was much less severe than in the spleen recipients.

The C57BL/10J mice in Group C, exposed to 750 rads, received spleen homogenates from the C3H donors. They uniformly developed amyloidosis, following 4 azocasein injections with features virtually identical to those found in similar C3H recipients (Group A).

The results (Text-fig. 3) thus demonstrate amyloid induction in X-irradiated, isogeneic or allogeneic recipients of either sensitized donor spleen cells or homogenates, or serum, immediately following 4 daily injections of azocasein. The recipients of the spleen developed much more extensive amyloidosis than recipients of the serum.

Spleen cells or homogenates from azocasein-treated C3H donors were injected into 2 groups of irradiated recipients (Text-fig. 4). Group A,
consisting of 5 and 16 mice exposed to 400 and 750 rads, respectively, failed to show amyloidosis after 2 azocasein injections. Group B, consisting of 5 and 7 mice exposed to 400 and 750 rads, respectively, also failed to develop amyloidosis after 4 injections of sodium bicarbonate.

Although recipients of sensitized spleen tissues developed amyloidosis after 4 injections of azocasein (Text-fig. 3), the results summarized in Text-fig. 4 show that 2 injections of the same antigen, or 4 of sodium bicarbonate were insufficient for amyloid induction in similarly prepared recipients.

Discussion

In a previous study, the induction time for amyloidosis by azocasein was markedly shortened in lethally irradiated C57BL/10J mice which had received intact spleen cells from isogeneic donors sensitized to azocasein. In contrast, no amyloidosis developed in recipients of spleen cells from nonsensitized donors. It was concluded that the transferred cells of the sensitized donor played a rate-limiting role in amyloidogenesis. The present results confirm these findings in a different mouse strain; in addition, they clearly demonstrate similar results with transfer of non-viable spleen cells or of serum, thereby ruling out the possibility that, under the conditions of these experiments, the intact or viable donor cells directly elaborated amyloidosis. The constant spatial relation of amyloid fibers to the RE cells in the recipients, also noted earlier and confirmed here, now becomes more meaningful. It is therefore concluded that amyloidosis resulted from an interplay between a subcellular factor derived from the lymphoreticular cells of sensitized donors and the fixed RE cells of the recipient, and that the latter cells determined the final location of the amyloid fibers.

While these investigations were progressing, the results of a somewhat similar study were reported by Werdelin and Ranløv. They transferred spleen cells from casein-sensitized mice into isogeneic recipients, which, after treatment with nitrogen mustard, showed splenic amyloidosis. It was initially concluded that the transferred cells both colonized the recipient and then elaborated the amyloid. Later studies by these authors have shown, however, similar results with transfer of nonviable cells.

Nature of the Donor Cell Factor

Although the donor cell factor will be referred to in the singular sense, it is acknowledged that more than one component could be involved in the proposed interplay.
Both the cellular source and the physicochemical identity of the donor factor remain to be explored. The results with the use of sensitized donor serums suggest, however, that the factor may be a soluble substance which can gain access to the systemic circulation from the cells of origin. Since the factor formed in response to injections of an antigen and was recoverable from the spleen (and lymph nodes⁹), the possibility that it is a product of immune reactivity of the donor's lymphoreticular cells therefore cannot be ignored. Further studies will be required to determine if such a product is a specific immunoglobulin which may or may not be complexed with the antigen, or a physicochemically related protein lacking antigen specificity, e.g., paraproteins, or some other material. Whatever its exact nature, the present study clearly shows that heavy antigenic challenge in mice results in the formation of a factor with amyloidogenic activity in appropriate recipients; the factor is (1) found in and transferrable by intact or disrupted spleen cells, or by serum; (2) stable at −20° C. for at least 3 months; and (3) active across allogeneic histocompatibility barriers—i.e., C₃H→C57BL.

Role of RE Cells in the Proposed Interplay

**Interplay Between RE cells and Factor.** A spatial relationship of amyloid deposits to the fixed RE cells of recipients was consistently observed. This indicates, at the very least, that RE cells play a dominant role in determining the final sites of amyloid deposition. The mechanism is as yet unknown, but at least three pathways can be considered: (1) the factor interacts, extracellularly, at or near the surface of RE cells with some material elaborated by the latter to form insoluble amyloid precipitates; (2) the sensitized factor is taken up by RE cells through endocytosis and then either causes, coordinates, or is permissive of amyloid synthesis by the RE cells; and (3) the effect of the factor is simply one of RE cell stimulation, which may be additive to other RE cell changes involved in amyloid formation.

Kennedy has proposed⁹ that experimental amyloid is formed in a manner analogous to the first pathway. A hypothesis similar to the second was proposed by Menkin after he observed amyloidosis in rabbits that had been given injections of a euglobulin recovered from turpentine-induced pleural exudates in dogs: that the euglobulin affected the "metabolism" of RE cells in some manner that caused them to form amyloid.¹⁰

The case for the third pathway must be viewed in the light of the fact that experimental amyloidosis can be provoked by many dissimilar antigens—a fact that has generated numerous proposals¹¹ as to the role of
immunologic mechanisms in amyloidogenesis. However, those proposals which invoke the necessary participation of specific immunoglobulins or of a specific immune mechanism \(^{12}\) for amyloidosis actually raise a paradox which they fail to explain, i.e., the association of specific immune reactivity with a nonspecific response—amyloid. In addition, recent observations in mice,\(^{4,13-15}\) chickens,\(^{16}\) rabbits,\(^{17,18}\) and humans\(^{19}\) have indicated that amyloidosis may occur spontaneously or be readily induced by soluble protein antigens under conditions that were either conducive to or causative of states of partial or generalized immune unresponsiveness. It is of considerable interest that these states may sometimes be accompanied by hyperplasia\(^{14,16,18}\) and/or an increased phagocytic function of RE cells,\(^{20}\) and the importance of RE cell stimulation in amyloidosis has already been emphasized by many workers, especially Teilum.\(^{19}\)

Considered in light of the information provided by the model reported here, amyloid induction by large amounts of different and widely dissimilar antigens may involve the formation of a product by the lymphoreticular cell population; such a product, although possibly an immunoglobulin,\(^{6}\) may not necessarily show antigen specificity. Alternatively, specificity may be a characteristic, and antigen-antibody complexes with antigen excess are formed. In either case, the product serves as only one means whereby a final common pathway for amyloid formation, probably RE cells,\(^{11}\) is stimulated or set into play. The case for an immunoglobulin which lacks antigen specificity is analogous to the induction of amyloid in recipients of homogenates of nonviable plasma cell tumors,\(^{21}\) the latter being associated with a paraproteinemia in intact states.

**Conditions Possibly Required in the Recipients.** X-irradiated recipients of sensitized spleen tissues failed to develop amyloidosis when given injections of sodium bicarbonate instead of azocasein. Since the latter was used to sensitize the donors, it may appear at first glance that transfer of some specific antigen-recognition immune process (adaptive immunity) was operational as an explanation of these differences. The reason for discounting this was previously discussed.\(^{1}\) It is noteworthy, in addition, that antigen injections of recipients were started 24 hr. after the latter were sublethally or lethally X-irradiated. It is possible, therefore, that the one effect of the antigen was nonspecific stimulation of RE cells, which are highly radioresistant. Further studies involving injection of recipients with different antigens and nonantigenic stimulants of the RE system are clearly required before this question can be resolved.

**Summary and Conclusion**

Inbred C\(_b\)H mice were injected daily with a soluble protein antigen, azocasein, for a period of time that fell short of that required for the de-
velopment of amyloidosis. Spleen, either as intact cells or as crude, frozen-thawed homogenate, or serum from these mice was transferred into sublethally or lethally X-irradiated isogenic or allogeneic recipients. The latter developed amyloidosis following a much shorter sequence of injections with azocasein. The amyloid deposits were spatially related to fixed RE cells. No amyloidosis developed in recipients of nonsensitized spleen tissues or serum.

These results confirm previous studies indicating that lymphoreticular cells of sensitized donors play an important rate-limiting role in amyloidogenesis, and show that nonviable cells or serum have the same capacity. It is concluded that amyloidosis resulted from an interplay of a subcellular product from lymphoreticular cells of sensitized donors with the RE cells of the recipients, and that the latter cells, at the least, determined the final location of amyloid fibers. The possible nature of the components and features of the interplay are briefly considered.

References


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