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TRANSFER AMYLOIDOSIS

STUDIES ON THE NATURE OF THE AMYLOID INDUCING FACTOR IN A MURINE TRANSFER SYSTEM

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SUMMARY

Transfer of amyloidosis between syngeneic mice was attempted with whole spleen cells and homogenates, nuclei, cytoplasmic fractions and various preparations of nucleic acids. Amyloidosis could be transferred from casein-treated donor mice by means of spleen cell suspensions, homogenates or crude nuclei, but not with nuclei treated with DNase, cytoplasmic fractions or five different nucleic acid samples, derived from whole spleens or crude nuclei. The transfer activity of crude nuclei was lost after storage at 0 or −20°C. The experiments indicated that the amyloid inducing factor is a labile, complex substance, possibly a nucleoprotein.

INTRODUCTION

Amyloidosis has been transferred between syngeneic mice by means of whole, viable, spleen cells (Werdelin & Ranløv, 1966; Hultgren, Druet & Janigan, 1967), and in 1967 Ranløv was able to transfer amyloid disease with spleen cell extracts from casein-treated mice (whole spleen homogenate, nuclear fraction). Based on this and other experiments Ranløv extended Teilum's two-phase theory (1954) of the pathogenesis of amyloidosis in the following way. In principle, amyloid formation involves two entirely different series of events: (1) a pyroninophilic tissue phase which can be caused by a number of different stimuli, though mainly (probably only) antigenic, acting via cell-mediated immunity; an actual amyloid phase, during which the amyloid substance is being deposited in the tissue by active secretion from cells belonging to the reticulo-endothelial system. The link between these two phases was believed to be a nucleic acid 'transfer factor' released from the pyroninophilic cells either directly or as the result of cell fragmentation. This transfer factor will impose an aberrant protein synthesis on RE cells resulting in amyloid formation (Ranløv, 1968).

The present work was performed in order to further characterize the above-mentioned 'transfer factor'.

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MATERIAL AND METHODS

All animals were derived from our inbred colony of the C3H strain, were between 8 and 10 weeks of age at the beginning of the experiment, and weighed 23–27 g. Sex distribution was equal.

Donors. 500 mice were given each a daily injection of 0·5 ml of a 5 % solution of sodium caseinate subcutaneously for 21 days. In addition 400 untreated control donors were included.

Cell suspensions. The spleens were gently teased apart in a loose-fitting Potter-Elvehjem glass homogenizer. After filtration through a metal sieve the suspension was washed three times in ice cold Hanks' BSS. The donor cell suspension was adjusted with Hanks' solution to a final concentration of 200×10^6 viable nucleated cells per ml.

Cell homogenates. Spleen cell suspensions were exposed to ultrasonic irradiation for 45 sec using a M.S.E. generator, frequency 20 kc/s, output 60 W. 1 ml of the resulting homogenate thus represented 200×10^6 nucleated cells.

Preparation of crude nuclei and cytoplasma. To 1 vol of a suspension of 200×10^6 spleen cells per ml in Hanks' solution was added 0·04 vol of 100 mM disodium–EDTA and 4 vol of distilled water. Subsequently the suspension was frozen and thawed twice. Isotonicity was re-established by the addition of 0·081 vol of ice-cold 2 M NaCl. The suspension was filtered through a double layer of cheese-cloth and centrifuged at 0°C at 900 *g* for 5 min. The pellet is referred to as crude nuclei, the supernatant as crude cytoplasm. The crude nuclei were suspended in 3 vol of Hanks' solution.

Crude nuclei were also prepared by the following two methods: (1) by homogenization in a close-fitting glass homogenizer of a suspension of cells in a hypotonic Tris-buffer (0·01 M NaCl, 0·0015 M $MgCl_2$, 0·01 M Tris-HCl, pH 7·4), with subsequent restoration of the isotonicity by addition of hypertonic Tris-buffer (0·8 M NaCl, 0·05 M $MgCl_2$, 0·01 M Tris-HCl, pH 7·4); (2) by suspending a pellet of cells in about 10 vol of a solution of 0·5% Tween 40 in a hypotonic Tris-buffer at 0°C for 5 min with subsequent restoration of the isotonicity. The nuclei were washed several times with Hanks' solution. Electron and light microscopy of the nuclear preparations obtained by these three methods revealed no significant differences—no intact cells were seen.

DNase-treated crude nuclei. Crude nuclei from a cell suspension with a packed volume of 100 μl were washed and suspended in cold hypotonic buffer (see above), which was made 1% and 0·5% with respect to Tween 40 and sodium deoxycholate. After a few seconds' shaking the pellet was washed and resuspended in hypertonic buffer (see above), and digested with 50 μg pancreatic DNase (Worthington) for 75 sec at 37°C.

Preparation of whole cell RNA. The spleens were immediately removed from anaesthetized mice, placed on dry ice, and cut into pieces of about 10 mm^3. Phenol extractions were carried out in two different ways: *Hot extracted RNA* was extracted by use of phenol and potassium phosphate buffer as described by Frederiksen, Tønnesen & Hellung-Larsen (1971). However, the extraction was performed at 55°C in the presence of 0·5% sodium dodecyl sulphate (SDS). The nucleic acids in the aqueous phase were re-extracted at 0°C in phosphate buffer without detergents (Hellung-Larsen & Frederiksen, 1972). The RNA was precipitated with 2½ vol ethanol and 0·1 vol 'saline' (Frederiksen *et al.*, 1971) and dissolved in Hanks' solution containing potassium polyvinyl sulphate (2 μg/ml). Hot extracted RNA which was not re-extracted contained about 60% RNA and 40%

DNA. Shortly before the injection of this material the suspension was homogenized using an Ultra Turrac to facilitate the injection of the DNA present. In some experiments diethylpyrocarbonate, a very potent RNase inhibitor (Solymosy *et al.*, 1968) was added to the frozen spleens (0·1 μg/mg). *Cold extracted RNA* was obtained by use of phenol-Tris-buffer with 1% SDS at 0°C as described elsewhere (Hellung-Larsen, Frederiksen & Plesner, 1972) or by use of phenol–phosphate buffer without detergents at 0°C as described by Frederiksen *et al.* (1971).

Preparation of RNA from spleen cell homogenates, crude nuclei and crude cytoplasm. These three different fractions were extracted by the methods of cold extractions described above.

Evaluation of RNA sample. The extraction efficiency of the different procedures with respect to RNA varies from about 70% to about 90%. The composition and possible degradation of nucleic acid sample was judged by analysis of profiles from electrophoresis on 3% and 10% polyacryl-amide gels formed essentially as described by Loening with slight modifications (Hellung-Larsen & Frederiksen, 1971, 1972). The gels were scanned at 254 nm in a Joyce-Loebl UV-scanner. None of the cold extractions showed any signs of degradation, but the hot extracted whole cell RNA showed slight degradation of the ribosomal RNA components.

Incubation. In some of the experiments 'crude nuclei' were incubated with Hanks' solution at 37°C for 2–3 hr.

Recipients. Randomized syngeneic C3H mice were treated with injections of spleen cells or their fractions (Table 1). Fractions derived from 10^8 spleen cells were injected into each recipient. The recipients were injected either i.v. or i.p. as no differences were noted between these two routes of administration. After the transfer all recipients were treated with nitrogen mustard (Erasol®) 0·05 mg subcutaneously on days 0, 3 and 5, in order to accelerate amyloid formation (Teilum, 1954). Controls were also treated with spleen cell injections or their fractions derived from normal donors. All control animals received nitrogen mustard of the schedule used for experimental animals.

The day after the last nitrogen mustard injection, all recipients were killed. The spleen, liver and kidney were fixed in neutral formaline. Sections were stained with haematoxylin-eosin, methyl green–pyronin, alkaline Congo Red, and PAS stain. Amyloid was identified by its morphology and by its birefringence with Congo Red under crossed polarizing filters. The degrees of amyloidosis, if any, were evaluated on sections of spleens according to the semiquantitative method—ranging from 0–6—described by Christensen & Hjort (1959).

RESULTS

The results are shown in Table 1. Spleen cells and their homogenates consistently transferred amyloidosis provided the donors were casein-treated prior to transfer. Furthermore, it was possible to obtain comparable positive transfer results with injections of crude nuclei. Treatment of the crude nuclei with DNase for a very short period led to complete loss of the transfer capacity. Only a few of the recipients of crude cytoplasmic fraction from experimental animals developed amyloid to a minor extent in the spleen. The negative result with cytoplasmic fractions could be due to a dilution of the transfer factor. It was therefore decided to make a few experiments with 2–3 times concentrated crude

cytoplasm. These experiments, however, were also negative. Incubation of crude nuclei 37°C for 2 hr or storage at 0 or 20°C for 3 days resulted in complete loss of the transfer activity. The table further shows that none of the nucleic acid samples could induce amyloidosis in the recipients. It is noteworthy that no recipients having received material from untreated donor mice developed amyloidosis.

TABLE 1

	No. of recipients		Incidence of amyloid		Average degree of amyloid in the spleens of the recipients	
Transfer of:	Experimental	Control	Experimental	Control	Experimental	Control
Spleen cell suspension	60	60	56/60	0/60	2	0
Homogenates	30	20	25/30	0/20	2	0
Crude nuclei*	30	20	22/30	0/20	1	0
DNase treated crude nuclei	5	5	0/5	0/5	0	0
Crude cytoplasm	12	9	2/12	0/9	0	0
Concentrated crude cytoplasm†	3	3	0/3	0/3	0	0
Incubated crude nuclei	15	10	0/15	0/10	0	0
RNA in whole cells (cold extraction)	4	4	0/4	0/4	0	0
RNA+DNA in whole cells (hot extraction)	4	4	0/4	0/4	0	0
RNA in spleen cell homogenate (cold extraction)	4	4	0/4	0/4	0	0
RNA in crude nuclei (cold extraction)	4	4	0/4	0/4	0	0
RNA in cytoplasm (cold extraction)	4	4	0/4	0/4	0	0

* The transfer activity was abolished after storage at −20°C or at 0°C for 3 days.
† Crude cytoplasm concentrated 2–3 times by suction-dialysis.

DISCUSSION

The transfer of amyloidosis cannot be explained solely as the result of cellular immune reactivity. This requires live cells (see Paterson, 1966) and unlike amyloidosis can be transferred by thoracic duct lymphocytes (Clerici *et al.*, 1969) or lymphoid cells from lymph nodes, peritoneum, thymus and bone marrow (Hardt, 1971). Thus the transfer of amyloidosis differs from that of classical cellular immunity as only the spleen is active, and it has been suggested that amyloidosis may be due to infection (Muckle, 1968; Clerici *et al.*, 1969). However, the bacterial pathogenesis has been ruled out by Claesson & Hardt (1972), who have shown that germ-free mice develop amyloidosis regularly after repeated injections of sterilized casein. A virus aetiology cannot be ruled out, but the data from Table 1 show that transfer of crude nuclei results in approximately the same degree of amyloidosis as transfer of suspensions of whole cells, thereby indicating that a possible virus need to be localized in the nucleus.

Ranløv (1967) has shown that nuclear fractions of spleen cell homogenates derived from casein-treated donors are effective in initiating a rapid amyloidogenic process in healthy recipients. In the present work we have been able to transfer amyloidosis with nuclei prepared by three different methods. However, any further treatment of our crude nuclei left them inactive. Neither Ranløv nor we were able to transfer amyloidosis with the cytoplasmic fraction of the above-mentioned homogenates, and this makes it unlikely that the transfer factor of Lawrence's is involved in the process.

The recent work on the role of nucleic acids in immune processes (see Pleseia & Braun, 1968) led us to examine whether various preparations of nucleic acids were capable of transferring amyloidosis in the present system. Although the protein-free nucleic acid preparations had no such effect, it does not rule out an instable nucleoprotein as the amyloid inducing factor (AIF). The instability of the amyloid inducing factor in the crude nuclei, with regard to resistance towards incubation with or without enzymes and with regard to storage at low temperatures, makes this assumption likely.

After stimulation with the specific antigen sensitized lymphocytes release a number of factors (lymphokines), which can either react by stimulating other lymphocytes (transfer factor, lymphocytotoxic factor, blastogenic factor) or by altering macrophage function (migration inhibition factor, macrophage aggregation factor). During the antigenic stimulation with casein used in the experimental induction of amyloidosis the lymphocytes proliferate (the pyroninophilic phase) and produce humoral antibodies (Giles & Calkins, 1958) as well as cellular immune reactivity towards casein (Ranløv & Hardt, 1970; Cathcart, Mullarkey & Cohen, 1971). Tentatively, we therefore propose that lymphoid cells, after prolonged and continued antigenic stimulation, release a substance of nuclear protein nature which, in a complex with the specific antigen, forms the amyloid inducing factor. This amyloid inducing factor acts on the intercellular level by stimulating the adjacent macrophages (immobilized in juxtaposition by the concurrent MIF) to produce an abnormal glycoprotein : amyloid.

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