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Evidence for a Thy-1-Like Molecule Expressed on Earthworm Leucocytes

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ABSTRACT—In this study, we analyzed the presence of a Thy-1 homolog in the earthworm, *Lumbricus terrestris*, using several monoclonal and xenoantisera in indirect immunofluorescence (IF) assay. The reactivity of monoclonal antibodies Thy-1.1 and Thy-1.2 proved specificities to the Thy-1.1 determinant. A rabbit anti-rat Thy-1 (Thy-1.1) antiserum was further investigated in IF assay by *in vitro* quantitative absorption. In all assays, earthworm leucocytes inhibited reactivity of antiserum as effectively as rat thymocytes in contrast to BALB/c (Thy-1.2) thymocytes. Thus, a Thy-1.1 cross-reacting determinant is probably expressed by a Thy-1 homolog on leucocytes of earthworms. The serological similarity between the earthworm Thy-1 homolog and the Thy-1 molecule in vertebrates will be strengthened by future immunochemical data.

INTRODUCTION

Coelomic fluid of earthworms contains several morphological categories of leucocytes which have been shown to play prominent role in allogenetic and xenogeneic graft rejection; the case of certain leucocytes associated with class I antigen in mammals [cf. in 1]. But until now, no attempts has been made to elucidate the nature of leucocyte membrane structures invovled in such phenomena. Recently, Roch *et al.* [2] succeded in demonstrating serological evidence for a membrane structure related to human β_2-microglobulin expressed by certain earthworm leucocytes. In searching for the origin of Thy-1, Shalev *et al.* [3] have demonstrated Thy-1-like molecule in total extracts of earthworms and several other invertebrates by using radioimmunoassays. Their analysis did not involve, however, a search for a Thy-1-like molecule in association with certain earthworm leucocyte membranes, which we have demonstrated in the present study. The phylogeneic studies of a molecule may contribute to increased understanding of its function and significance. The function of Thy-1 is still unknown but several lines of evidence suggest that the molecule is involved in T cell activation [4]. Recent reports strongly suggest that Thy-1 shares amino acid homolog with the constant and variable regions of immunoglobulins (Ig), with beta-2-microglobulin and major histocompatibility complex (MHC) encoded antigens [5, 6]. The hypothesis that either the Thy-1 or the β_2-microglobulin gene are representatives of the ancestral gene must be supported by evidence of high evolutionary conservation of these genes or molecules [7]. Thus, it is of vital importance to reveal more concerning the Thy-1 molecule in invertabrates and to trace its evolutionary origin and function.

In this study, we analyzed the presence of the Thy-1 homolog on earthworms (*Lumbricus terrestris*) utilizing several monoclonal and xenoantisera in indirect immnofluorescence assay. We observed a strong cross-reaction in which both anti-rat Thy-1.1 and anti-mouse Thy-2 monoclonal antibodies detected a molecule, on the cell surface of earthworm leucocytes suggesting the oocurrence of cell surface-bound forms of a putative Thy-1 homolog at this level of evolution.

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MATERIALS AND METHODS

Animals

About 200 earthworms, *Lumbricus terrestris* (Lumbricidae, Annelida) exhibiting secondary sexual characteristics were purchased from Sure-Live Meal Worm Co., Torrance, CA, and maintained at 12°C in natural soil in the laboratory. Male and female, 5 to 7 weeks old, BALB/c mice, Wistar rats and New Zealand white rebbits were obtained from the United State Naval Medical Research Unit No. 3, Cairo.

Cell suspensions

L. terrestris leucocytes were harvested by submerging worms in 10% alcohol solution as described previously [1]. After worms shed leucocytes through integumentary pores at room temperature (22°C) the leucocytes were decanted into fresh Ca^{2+}, Mg^{2+} free buffered saline solution (BSS: 10 mM KH_2PO_4, 10 mM K_2HPO_4, 0.11 M NaCl, 10 mM HEPES; pH 7.2) through a stainless steel screen to remove debris. Pools of leucocytes from at least 30 worms were used, washed by centrifugation at $450 \times g$ for 5 min at 4°C, and their viability assessed by trypan blue exclusion [8]. Suspensions of rat and BALB/c mouse thymocytes were prepared by teasing the thymus in PBS, pH 7.2. After washing 3 times each for 5 min by centrifugation at $600 \times g$, thymocytes were counted and their viability assessed by trypan blue exclusion.

Reagents

Rabbit anti-purified rat Thy-1 antiserum were kindly provided by Dr. M. H. Mansour (University of California, Los Angeles). Previous findings indicate that rabbit-anti-rat Thy-1 antiserum recognizes three antigenic determinants: the rat-specific Thy-1 xenoantigen, the rat-mouse cross-reacting xenoantigen, and the Thy-1.1 determinant [9]. BALB/c mouse anti-rat Thy-1 (ascites fluid, IgG MRCOX-7 anti-rat Thy-1.1 mAb) was purchased from Accurate Chemical Scientific Corporation, Westbury, NY. BALB/c mouse anti-AKR/J mouse thymocytes (ascites fluid, 7S IgG anti-mouse Thy-1.1 mAb) and AKR/J mouse anti-C3H thymocytes (19S IbM anti-mouse Thy-1.2 mAb) were purchased from New England Nuclear, Boston, MA. Rabbit anti-BALB/c mouse brain serum and rabbit anti-C3H mouse brain serum were purchased from Bionetics Laboratory Products, Kensington, MD and absorbed locally with crude liver cell-membranes from BALB/c and C3H mice. Normal rabbit and rat sera were collected in the laboratory from unimmunized animals. Fluorescein isothiocyanate (FITC-) labelled goat Ig, anti-rabbit globulins were purchased from Behring Institue, Marburg, West Germany and FITC-labelled rabbit anti-mouse Ig from GIBCO, Grand Island, NY.

Indirect immunofluorescence (IF) assays

Analysis of different specificities in the anti-sera against target earthworm leucocytes and/or rat and BALB/c mouse thymocytes was measured in indirect immunofluorescence assay by quantitative absorption. Preliminary experiments, performed to define optimal labelling conditions for our system, indicated that fluorescent antibodies must be used at a dilution of 1 : 20 for the anti-mouse Ig and 1 : 25 for the anti-rabbit globulins. Apart from appropriate changes in target cells, antisera and anti-serum dilutions (details are further elaborated in the Result Section), the standard assay involved absorption of the first-step antibody (200 μl) with increasing numbers of earthworm leucocytes and/or rodent thymocytes for 16 hr at 4°C, followed by incubation with $1\text{–}2 \times 10^6$ freshly-prepared target earthworm leucocytes and/or rodent thymocytes for 45 min at 4°C. After 3–4 washings in PBS, target cells were reincubated with 100 μl of fluorescent conjugate for an additonal 45 min at 4°C. After 3–4 washings, cells were finally mounted on microscope slides, scored alternately in phase contrast and fluorescence microscopy. Percentage of positive labelled cells were determined by counting a minimum of 200 cells. Positive as well as negative controls, explained in the results, were included in each expeirment.

Iodination of cell surface and indirect immunoprecipitation of Thy-1 homolog

Aliquots of 10^7 viable earthworm leucocytes were surface labelled with 300 μCi $Na^{125}I$ by the

lactoperoxidase-catalyzed reaction [10]. Washed labelled cells were solubilized with 200 μl of 0.5% (w/v) Nonidet-P 40 in 0.15 M NaCl-0.02% NaN_3 (w/v-0.01 M Tris-HCl, pH=7.4), by incubation on ice for 1/2 hr. The cell particulates insoluble in NP-40 (nuclei and cellular debris) were removed by centrifugation at 1200×g for 35 min at 4°C. The supernatant containing solubilized cell surface components was used for indirect immunoprecipitation. To 10^7 labelled cells (200 μl), 50 μl of monoclonal anti-mouse Thy-1 were added. After incubation with antiserum for 45 min at 4°C, 100 μl of a 10% protein A bound to Sepharose 4 B (Pharmacia Fine Chemicals, Uppsala, Sweden) were added and incubated for an additional 45 min at room temperature. The precipitates were washed thrice with 0.2 M PBS, pH=7.2. Immunoprecipitates were dissolved in 2.2% SDS, 5% 2-mercaptoethanol, 0.1 mM EDTA in SDS-buffer and boiled for 3 min at 100°C. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% polyacrylamide gels. After electrophoresis, the gels were sliced (2 mm in length) and radioactivity counted in each slice by gamma counter [12].

RESULTS

The reactivity of both anti-Thy-1.1 monoclonal antibodies (mAbs Thy-1.1) and anti-Thy-1.2 monoclonal antibodies (mAbs Thy-1.2) was titrated in IF against earthworm leucocytes. The mAb Thy-1.1 showed a strong binding reactivity to earthworm leucocytes in contrast to mAb Thy-1.2 which was completely negative (data not shown). These results suggest that probably earthworm leucocytes are bearers of the Thy-1.1 determinant but lack the presence of the Thy-1.2 antigen.

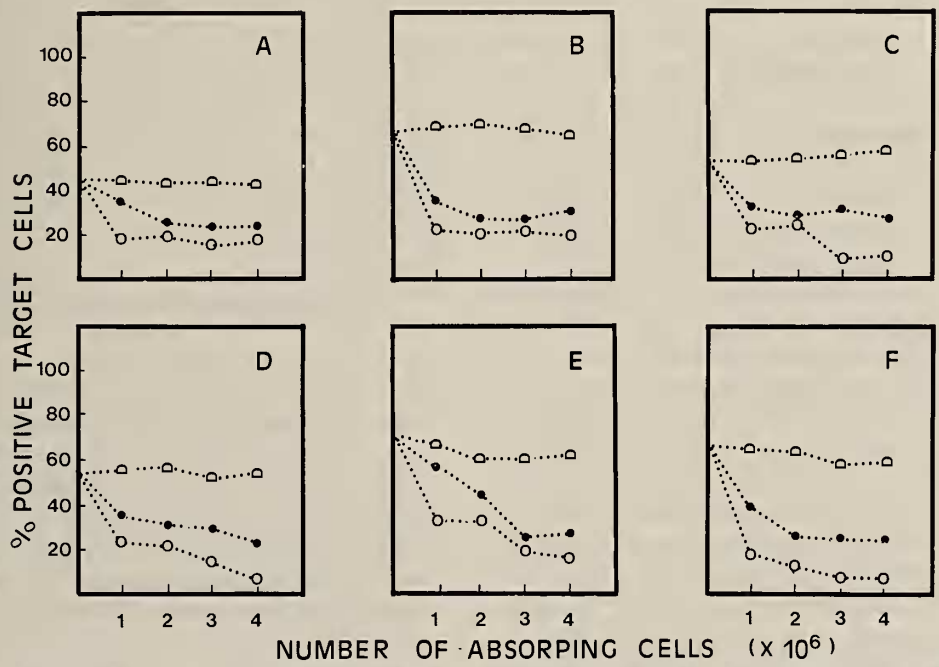

FIG. 1. Demonstration of a Thy-1 homolog on earthworm leucocytes using rodent Thy-1 antibodies in quantitative absorption assays. Numbers of absorbing cells are shown on the abscissae. Aliquots of earthworm leucocytes (●) and rat (○) and BALB/c mouse (□) thymocytes were used as absorbents to assay the activity of anti-rat Thy-1.1 mAb (diluted 1 : 1500 in the absorbing assay) to rat thymocytes (A) and earthworm leucocytes (B), the activity of anti-mouse Thy-1.1 mAb (diluted 1 : 1800 in the absorbing assay) to rat thymocytes (C) and earthworm leucocytes (D) and the reactivity of rabbit anti-rat Thy-1 antiserum (diluted 1 : 100 in the absorbing assay) to earthworm leucocytes (E) and rat thymocytes (F). Each point in the curves represents the mean value of two separate experiments.

Recent amino acid sequence data of murine brain derived Thy-1 molecules have demonstrated that protein encoded by the allels Thy-1.1 and Thy-1.2 differ by one amino acid residue, therefore antibodies are directed exclusively to one allelic Thy-1 product. Bearing this in mind, it was necessary to perform a set of quantitative absorptions to be certain that anti-Thy-1.1 mAb indeed detects the earthworm putative Thy-1 homolog. The absorptive capacity of worm leucocytes, rat and BALB/c mouse thymocytes to reduce the activity of BALB/c anti rat Thy-1 mAb towards rat thymocytes and earthworm leucocytes is depicted in Figure 1A and B, respectively. Within the given range of absorbents, earthworm leucocytes reduced detectable reactivities of mAb Thy-1.1 determinants as effectively as did the absorption of rat thymocytes (Thy-1.1 strain). In contrast, the binding capacity of mAb was not completely abolished by BALB/c mouse thymocytes (Thy-1.2 strain). Significant absorptions were further confirmed in quatitative absorption assays using BALB/c mouse anti-AKR/J mouse thymocyte mAb with target rat thymocytes (Fig. 1C) and earthworm leucocytes (Fig. 1D). This resulting pattern of reactivity was similar to that observed using anti-rat mAb, confirming the detectability of a Thy-1.1 cross-reacting determinant, by a putative Thy-1.1 homolog on earthworm leucocytes.

Although results from quantitative absorption assays suggest that the Thy-1.1 epitope is shared between rat Thy-1 molecules and a putative Thy-1 homolog on earthworm leucocytes, there is no evidence that the molecule(s) are serologiacally identical or not. In order to further map the difference between rodent and earthworm Thy-1, rabbit anti-rat Thy-1 antiserum was absorbed with rat and BALB/c mouse thymocytes and assayed with rat thymocytes (Fig. 1F) and earthworm leucocytes (Fig. 1E) as target cells. While rat thymocytes are capable of absorbing nearly all the binding reactivity, BALB/c mouse thymocytes absorbed only 30% and earthworm leucocytes absorbed 70% of the reactivity. Specific absorption occurred using the same number of earthworm leucocytes and rat thymocytes which indicates two main antibody specificites: a specificity directed to the antigenic determinants shared by earthworm and rat (mouse), the earthworm-rat (-mouse) cross reacting xenoantigenic determinant and a specificity directed to an antigenic determinant selectively absorbed by rat, the Thy-1.1 antigenic determinant. In contrast, reactivity of rabbit anti-rat Thy-1 against BALB/c mouse thymocytes as targets was completely diminished by BALB/c mouse thymocytes and earthworm leucocytes, (data not shown); rat thymocytes abosorbed only about 28% of the antibody specificities.

Immunoprecipitates of earthworm ^{125}I-labelled, solubilized leucocytes were obtained by anti-mouse Thy-1.1 mAb and subjected to SDS-PAGE analysis using 10% gels under reducing conditions. The results indicate an apparent molecular weight of 28.2 KD for the Thy-1 homolog (Fig. 2).

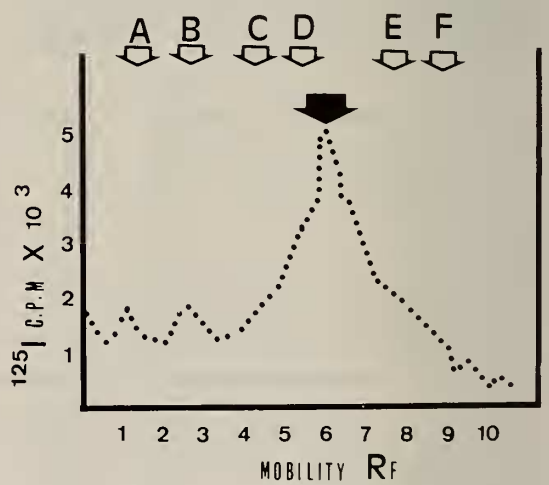

Fig. 2. Polyacrylamide gel electrophoresis (PAGE) analysis of immunoprocipitated earthowrm leucocytes. The position of protein markers that we run simultaneously and stained with Coomassie Blue is indicated by arrows (A, phosphorylase b (94.0); B, BSA (67.0); C, ovalbumin (43.0); D, carbonic anhydrase (30.0); E, Soybean trypsin inhibitor (20.0) and F, Lactalbumin (14.0). This indicates an apparent molecular weight of 28.2 KD for the Thy-1 homolog.

DISCUSSION

Although Thy-1 has been used as a T-cell marker, there is no difinite resolution as to its role in T-cell functions. Recently, it has been postulated that Thy-1 may be ancestral molecule from which

all members of the Ig superfamily might have evolved [11–13]. To follow up this ancestral molecule, studies in invertebrates, seemed to us, to be highly warranted. Although, invertebrates leucocytes have long been overlooked with respect to cell markers, they might prove to be the most suitable substrate for defining the roots of Thy-1 homolog from an evolutionary viewpoint.

In our approach to search for a Thy-1 homolog on earthworm leucocytes, the reactivity of two mAbs or proven specificities to the Thy-1.1 determinant of rat and AKR/J mouse Thy-1 molecule [14] and rabbit anti-rat Thy-1 antiserum [9] towards earthworm leucocytes was investigated in IF assays by quantitative absorption. Although, neither reagents generated a response the putative earthworm Thy-1 antigen, both were depleted of anti-Thy-1.1 reactivity by absorption with earthworm leucocytes to the same degree as rat thymocytes (Thy-1.1 strain). Due to the specificity of the antisera and the sensitivity of our assay, the membrane determinant revealed on earthworm leucocytes seemed to be related to the Thy-1 molecule. Two antibody specifities in the antisera were recognized and found to be directed towards two antigenic determinants expressed on earthworm leucocytes. These were referred to as: the earthworm-rat (mouse) cross reacting xenoantigenic determinants and the Thy-1.1 qantigenic determinant. This observation substantiates the occurrence of a Thy-1 homolog on earthworm leucocytes, which in terms of structure, might share a common pattern with rodent Thy-1 molecule, manifested by the expression of Thy-1.1 determinant.

The similarity of Thy-1.1 determinant in earthworm and rat, in particular, may be strengthened by results from immunoprecipitation assays. The estimated molecular weight was in stricking agreement with values obtained for rat [9], mouse [15], human [16], dog [16], frog [17] and tunicate [18] Thy-1 glycoprotein. However, the ultimate proof for this assumption would be obtained by biochemical characterization, purification and amino acid sequence studies on earthworm Thy-1 epitope which are in progress. The presence of another vertebrate-like molecule, within the Ig superfamily such as Thy-1, among earthworms may not seem surprising since our results add to a growing list of such shared molecules, including for example, β_2-microglobulin [4]. In terms of similarities in antigenicity, it is conceivable that the earthworm leucocytes Thy-1 homolog represents an ancestral Thy-1 molecule that underwent diversification. Vertebrates are assumed to have evolved from the chordate line represented by tunicates which are deuterostomes. That a putative Thy-1 homolog exists in earthworm which belongs to the protostome line, supports the view that Thy-1, a component of the Ig superfamily, is present universally. Moreover, this argues for later diversification of the terminal gene during evolution of other members of the superfamily such as Ig which is up to now not demonstrable in invertebrates and only present in all vertebrates [6, 7]. Such information may provide essential clues to our understanding of fundamental events concerning the evolution of the immune system.

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