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Production and characterization of a new monoclonal antibody, GR-ICOR-2, recognizing sarcomeric actin: analysis of the expression in the developing chick heart

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Summary. We produced and characterized a specific monoclonal antibody (mAB) designated GR-ICOR-2. This mAb recognizes sarcomeric actin molecules (43 kDa) and was used in an immunohistochemical analysis of staining patterns in Hamburger and Hamilton's stages 18, 22 and 25 (HH 18, 22 and 25) embryonic chick hearts. Staining showed a mainly cytoplasmic distrubition in three regions: the atrioventricular (AV) canal cushion tissue, the primitive ventricle, and conal crests. In addition, this mAb-cross-reacted with rabbit and human cardiac and skeletal muscle tissue; but not with smooth muscle tissue.

Key words: Actin, Embryo, Heart, Monoclonal antibody

Introduction

Actin, is one of the two largest proteins involved in cellular motility and contractility. Composed of a single 43 kDa polypeptide, it is the largest components of thin filaments. Actins are a family of six highly conserved proteins. Four of these six isoproteins, found in muscle cells, differ in fewer than nine amino acid residues of a total of 375. Because of their degree of conservation, antibodies have been produced (Lin et al., 1984; Skalli et al., 1986) four use in studies of the temporal and spatial distribution of these isoproteins.

In the adult heart, cardiac alpha-actin is the predominant isoform, whereas in skeletal muscle, skeletal alpha-actin predominates (Vanderckhove and Weber, 1979; Vanderckhove et al., 1986; Bennetts et al., 1986). Different isoforms of actin are also found in smooth muscle, e.g. smooth muscle α-actin, located mainly in vascular tissue, and smooth γ-actin, found predominantly in the gastrointestinal and genital tracts (Vandekerchkove and Weber, 1979). Although adult muscle tissue contains only one major type among the actin isoforms, in developing muscle several forms of actin are coexpressed (Hayward and Schwartz, 1986; Ordahl, 1986; Owens et al., 1986; Clowes et al., 1988; Hayward et al., 1988; Ruzicka and Swartz, 1988; Otey et al., 1988; Woodcok-Mitchell et al., 1988; Garner et al., 1989; Strauch and Reeser, 1989; Handel et al., 1989; Sawtell and Lessard, 1989; Babai et al., 1990).

During cardiogenesis in the chick embryo, actin isoforms have been analyzed only at the mRNA level (Ruzicka and Swartz, 1988). Recently, Sugi and Lugh (1992) used in situ hybridization to study the expression and localization of sarcomeric actin during avian heart development.

Certain research problems in the field of cardia morphogenesis, can be most effectively solved through the use of monoclonal antibodies against embryonic heart tissue (Prados et al., 1992, 1993). A considerable number of questions remain unanswered in cardiac embryology, and the enigmas multiply when attempts are made to elucidate the early structural arrangement of the myocardium and endocardium, and more specifically the degree of involvement of each of these tissues in the configuration and colonization of the cardiac jelly. Until recently, habitually employed investigate techniques were unable to shed light on such topics, as the data yielded by the most commonly used histological procedures were too inespecific in nature.

In this paper we present a mAB that reacts with sarcomeric actin, describe its distribution during cardiac development in the chick embryo, and report its cross-reaction with cardiac and skeletal muscle tissue of rabbits and humans.

Materials and methods

Embryos

Fertile White Leghorn (Shaver Starcross 288) chicken eggs were incubated at 37.5 °C in a standard incubator with 80% relative humidity. This strain has

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been previously shown to present a low rate of spontaneous mutations (Aránega et al., 1973, 1985). The hearts from embryos at different stages of development were classified according the staging system of Hamburger and Hamitlon (1951). A total of 200 chick embryos were incubated to Hamburger and Hamilton's stage 20 (HH 20). At this stage development was stopped to remove the hearts, which were used to raise monoclonal antibodies according to the following procedure: hearts were perfused and extracted under dissecting microscope (Wild Type 376788) in order to assure accuracy in ablation of the pericardium and levels of sectioning. The planes of section were determined based on the atlas of De La Cruz et al. (1972). Then the heart was separated cranial to the atrioventricular canal, excluding the more superiorly situated auricular zone, while the anterior limit was a plane cranial to the truncus arteriosus.

Immunization

Fifty embryonic thick hearts at HH 20 were haversted for each immunization and macerated with 2 ml phosphate-buffered saline (PBS) (pH 7.4) in a specially designed micromortar and pestle. This was followed by centrifugation at 1,500 r.p.m. for 10 min, and the resulting pellet was resuspended in 0.2 ml PBS. The immunization protocol, based on that described by Stahli et al. (1980), consisted of a series of intraperitoneal injections of approximately 100 µg of antigen emulsified in Freund's complete adjuvant in 2-month-old Balb/c mice. The first immunization was followed by two repetitions at weekly intervals, then a final injection three days after the penultimate inoculation, over a total immunization period of 24 days.

Cell fusion

The myeloma cell line used was non-immunoglobulin secretin Sp2/15 derived from the Pe-X63Ag8 strain (Fazekas De St. Growth and Scheideger, 1980). The lymphocyte subset was obtained from the spleens of mice previously immunized as described above. Hybridomas were cultivated in tissue cultures clusters, 50% of which contained a feeder layer consisting of an aliquot of 5 x 10^5 nonimmunized mouse spleen cells which had been teased apart with curved needls and resuspended in RPMI 1640.

Screetining of supernatants

Undiluted supernatants (20 µl) of growing hybridomas were screened for secreted antibodies by indirect immunofluorescence on cryotome sections (5 µm) of chick embryo hearts (HH 20). After screening, positive hybridomas were cloned by the limiting dilution technique (Springer, 1983). Ascitic fluids were obtained by intraperitoneal injection of a chosen clone to pristane-primed Balb/c mice.

Double immunodifussion

Subclasses of monoclonal immunoglobulins were identified by double immunodiffusion, using rabbit antisera to mouse IgG, IgG2a, IgG2b, IgG3, IgA and kappa and lambda IgM (Sigma, St. Louis, MO). GR-ICRO-2 was IgM-positive.

Western blotting

The mAb GR-ICRO-2 was further characterized by Western immunoblot according to Towbin et al. (1979). After polyacrylamide gel electrophoresis (12% separating gels) of total proteins of chick embryo heart (15 µg) and purified actin (0.01 µg) (Sigma), in the presence of sodium dodecyl sulphate (SDS) (Laemmli, 1970), the gels were transferred to nitrocellulose membranes (Bio Rad Laboratories, Richmond, CA) by running at 30 V overnight in 25mM Tris (hydroxy-methyl)-aminomethan-hydrochloridric (pH 8.3), 192mM glycine and 20% (v/v) methanol. The membranes were washed with 10mM Tris-buffered saline (pH 7.4) in 0.05% Tween 20, and incubated with the mAb at different dilutions (1/400, 1/600, 1/800 and 1/1000 for 3 h at room temperature. Positivity was detected with horseradish peroxidase-conjugated goat antibody to mouse IgM (Sigma), and developed with 4-chloro-naphthol as described in the protocol of the immunoblot assay kit (BioRad).

Tissue preparation

Fertile White Leghorn chicken eggs were incubated as described above, and the chick embryo hearts were removed at HH 18, 22 and 25. The hearts were snap-frozen in liquid nitrogen and mounted in OCT embedding medium for frozen tissue specimens (Tissue Tek, Miles Laboratories, Naporville, IL). Frozen tissues were cut into cryostatic sections (Slee cryostat, Giralt, Huntingdon, UK) measuring 5-7 µm in thickness (Bright FS/FAS, Huntingdon, UK), and the sections were dried under an air current for 4 5min. After fixation for 5-10 min in acetone at -4 °C, the slides were again dried for 15-30 min, wrapped in aluminium foil and stored at -20 °C until use.

Immunofluorescence staining

Frozen sections were incubated with the mAb GR-ICOR-2 at different dilutions (1/50, 1/100, 1/200 and 1/400 with ascites and 1/20, 1/40 and 1/80 with culture supernatant), 20 µl at room temperature in a humidified chamber for 45 min, then washed for 10 min in PBS. The second incubation (45 min), with a 1:50 dilution of fluorescein isothiocyanate-conjugated goat antimouse IgM (Sigma), took place at room temperature in a dark, humidified chamber, and was followed by a final washing in PBS. Controls were made using ascites containing isotype-matched irrelevant mAb. The slides

were mounted at pH 7.2 in FA aqueous mounting fluid (Difco Laboratories, Detroit, MI) for fluorescence microscopic observation under a Nixon HFXIIA light microscope designed for epifluorescence studies. Photographs were taken with a Nixon FX-35WA camera, using Kodak Tri-X Pan 400 ASA film at different exposure times.

Alkaline immunophosphatase anti-alkaline phosphatase

Serial cryostatic sections of embryonic chick heart (HH 8, 22 and 25) were allowed to thaw for 20 min, then incubated for 45 min in a humidified chamber with GR-ICOR-2 at dilutions of 1/50, 1/100, 1/200 and 1/400 with ascites and 1/20, 1/40 and 1/80 with culture supernatant. The slides were washed for 10 min with Tris-buffered saline and reincubated in the presence of a second antibody (rabbit antimouse immunoglobulin, Sigma) at 1/20 dilution in a mixture of 75% Tris-buffered saline and 25% human serum. After incubation for 45 min in a humidified chamber, the slides were again washed for 10 min with Tris-buffered saline. A third and final incubation was done with alkaline immunophosphatase anti-alkaline phosphatase at a 1/50 dilution for 45 min under idential conditions of temperature and humidity, and was likewise followed by washing with Tris-buffered saline for 10 min. Slides were developed with chromogen as follows: 2 mg napthol-Ag-MX-phosphate was dissolved in 0.2 ml dimethyl formamide in a glass test tube. To this was added 9.8 ml 0.1M Tris-buffered saline (pH 8.2). Immediately before use the mixture was dissolved in Fast red TR salt to a concentration of 1.0 mg per ml. 15.0 levamisole was also added to block the endogenous activity of alkaline imunophosphatase anti-alkaline phosphatase. The chromogen was filtered directly onto the slides and allowed to act for 15-20 min. Excess chromogen was removed by washing with Tris-buffered saline followed by tap water for 2-3 min. Nonstaining areas were counterstained with haematoxylin for 1 min. followed by washing under tap water and in distilled water. Controls were performed using the same methods as with indirect immunofluorescence staining. Finally, cover glasses were mounted with Apathy's solution, and the slides were examined under a light microscope.

Results

Characterization of the monoclonal antibody

Springer's technique (1983) was used to obtain a new mAb, designated GR-ICOR-2, that recognizes sarcomeric actin in embryonic chick heart tissue. The specificity of our mAb was checked with Western blot analyses. Ascitic of mAb-GR-ICOR-2 was tested at different dilutions, with the most effective reaction seen at a dilution of 1/800. When tested against total proteins from chick embryo cardiac cells, the mAb recognized a single 43 kDA molecule. The mAb also recognized sarcomeric actin (43 kDa) purified from rabbit heart (Sigma) (Fig. 1).

Immunohistochemical analysis

Our mAb was further tested by indirect immunofluorescence and alkaline immunophosphatase anti-alkalinephosphatase studies in HH 18, 22 AND 25 chick hearts to note differences in positivity between endo- and myocardium and cardiac jelly. Immunohistochemical assays showed GR-ICOR-2 to be a cytoplasmic marker. Cytoplasmic prolongations (podocytes) were also clearly stained and could be seen extending from cells which had colonized the cardiac jelly (Fig. 2a).

In HH 18 and 22 myocardiac cells were also positively stained although much more faintly than cell groups migrating in the extracellular matrix (Fig. 2a).

Indirect immunofluorescence and alkaline immunophosphatase anti-alkaline phosphatase assays revealed the presence of GR-ICOR-2 in nearly all dilutions tested, although the a 1/100 dilution of pure ascites was the most intensely positive. Pure ascite fluid on the other hand led to overstaining, and the 1/400 dilution was very weakly positive. Supernatants of mAb cultures were tested at different dilutions, with the most effective reaction seen at a dilution of 1/20. The picture in this

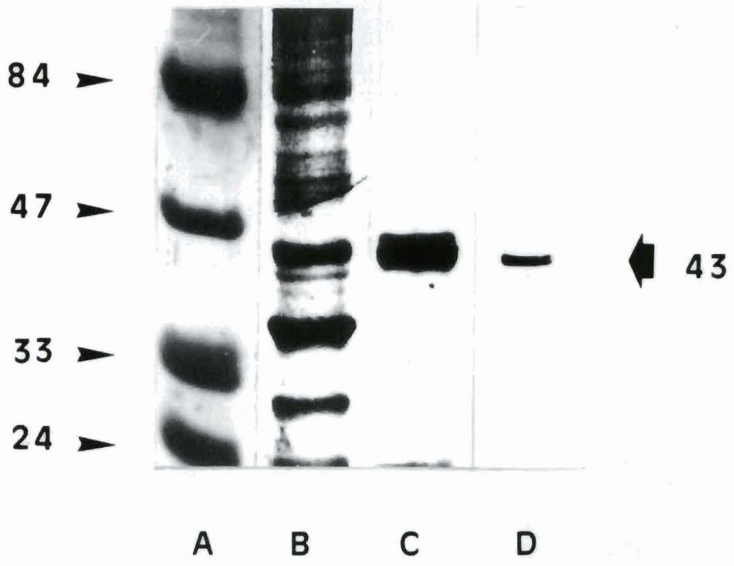

Fig. 1. Immunoblot analysis of the specificity of mAb GR-ICOR-2 toward sarcomeric actin from chick embryo cardiac cells. The cells were prepared as described in Materials and methods, and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). **Lane A:** prestained molecular weight controls (BioRad): soybean trypsin inhibitor, 24 kDa; carbonic anhydrase, 33 kDa; ovalbumin, 47 kDa; bobine serum albumin, 84 kDa. **Lane B:** total protein fraction from chick embryo cardiac cells obtained by SDS-PAGE. **Lane C**: total protein fraction (15 μg) from chick embryo cardiac cells. Immunoblotting with mAb GR-ICOR-2. **Lane D:** actin purified from chicken muscle (43 KDa) (0.01 μg) (Sigma). Immunoblotting with mAb GR-ICOR-2.

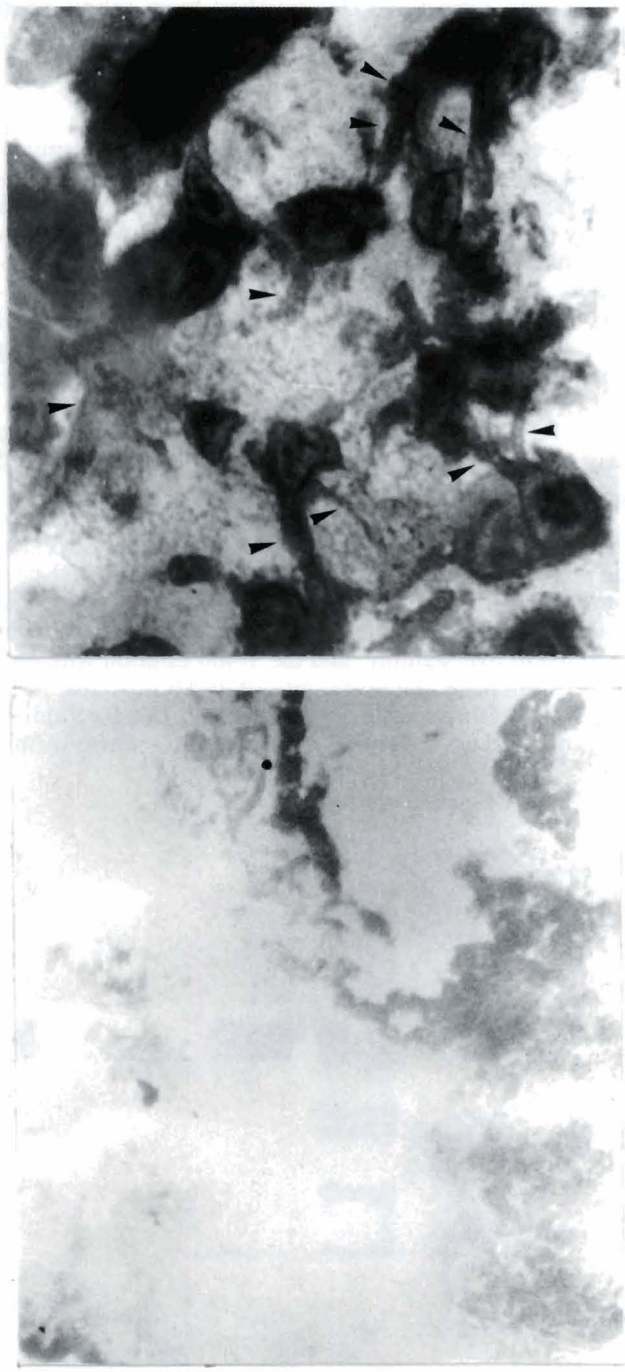

Fig. 2. a. Photomicrograph of myocardial cells stained with mAb GR-ICOR-2. This figure shows a characteristic voluminous center nuclei. Arrowheads points out the cytoplasmic prolongations (podocytes) in cells migrating through cardiac jelly. x 1,000. **b.** 5 µm thick cryostatic section of an HH 20 chick heart showing alkaline immunophosphatase anti-alkaline phosphatase technique results after exposure to mAb GR-ICOR-2. Positivity is seen mainly in the AV canal and dextrodorsal conal crests. x 100

case was similar to that seen with the 1/100 dilution of ascites.

Regional distribution of positive staining

Close examination of the stained regions in the HH 18 and 22 chick embryo heart showed positive zones located mainly at the level of the AV canal and the dextrodorsal crest within the conus (Fig. 2b). Positive staining was also seen in the posterior and caudal area of

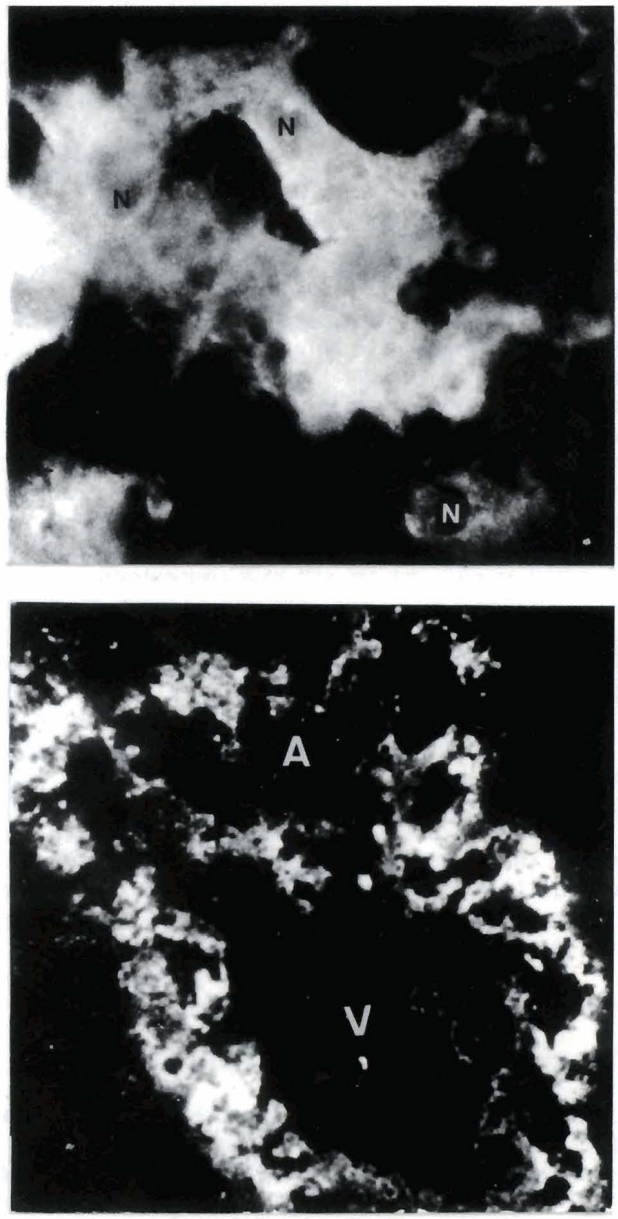

Fig. 3. a. Enlarged view of the AV canal cells. The cytoplasm shows a positive reaction in contrast to the nuclei (N). x 1,000. **b.** Sagital section of an HH 22 chick embryo heart, showing the auricular and ventricular pattern of distributions of mAb GR-ICOR-2. A: primitive auricle; V: primitive ventricle. x 100

the heart loop, while the remaining zones were not significantly positive.

In the AV canal (Fig. 3a) a large number of cells were positive. These cells often appeared in clusters interpersed with negative-stained cells. The areas pertaining to the dextrodorsal crests were most intensely stained at the conal level of the truncus arteriosus. Most cells in the extracellular matrix of the dextrodorsal crests showed evident cytoplasmic staining, which included cytoplasmic prolongations or podocytes, when these were present.

In HH 25, positivity was homogeneously distributed in the same regions as at HH 18 and 22 (Fig. 3b).

Table 1. Cross-reaction of monoclonal antibody GR-ICOR-2.

SPECIES	HEART	SKELETAL MUSCLE	SMOOTH MUSCLE
Chicken	+++	+++	-
Rabbit	+++	++	-
Human	+++	+++	-

+++: very strong staining; ++: strong staining; +: weak staining; -: negative.

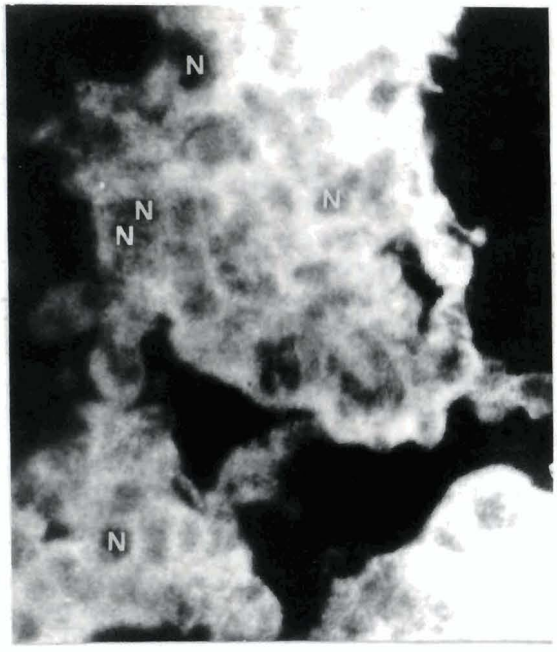

Fig. 4. a. Indirect immunofluorescence of the adult skeletal human muscle tissue, where are showed a intense expression of the antigens recognized by mAb GR-ICOR-2. x 100. **b.** This photomicrograph shows an intense staining of cytoplasm pattern by indirect immunofluorescence of adult rabbit skeletal muscle. x 200

Cross-reactions

Table 1 shows the cross-reactions with GR-ICOR-2 mAb. This mAb showed positive staining in rabbit and human cardiac and skeletal tissue. Smooth muscle was negative when incubated with GR-ICOR-2 mAb (Fig. 4a,b). On the contrary, no cross-reactivity was observed for the same type of tissues in mouse.

Discussion

The present study supports the characterization of mAb, named GR-ICOR-2 as an entity specifically recognizing cytoplasmic molecules identified as sarcomeric actin by Western blot analysis. Other mAbs against actin have been produced for the study, for cardiac myofibrillogenesis (Wang et al., 1988) and membrane attachment sites of actin microfilaments (Rogalski and Singer, 1985) or specifics to myofibrils (Kaehn et al., 1985). In embryonic chick heart tissue not all cytoplasm showed the same degree of positivity; staining was rather most intense in cushion tissue. Myocardial cells were also recognized, although staining was not as widespread or intense.

Indirect immunofluorescence assays showed less intense fluorescence in myocardial cells than in cells in the cardiac jelly. These latter are known for their migratory behaviour, although it has not been clarified whether they are of endocardial or myocardial origin.

Results obtained up to the present are insufficient to provide a definite explanation for the intense positivity seen with both indirect immunofluorescence and alkaline immunophosphatase anti-alkaline phosphatase in cells located in the cardiac jelly. Nevertheless, these cells showed a staining pattern similar to that in myocardiac cells, differing only in intensity, and hence suggesting myocardial origin. As an alternative hypothesis, it could be argued that if the migratory cells were of endocardial origin, they might possess specific antigens during their passage through the cardiac jelly. This notion is supported by the observation that such cells eventually differentiate, giving rise to a myocytic cell line, hence gradually acquiring the biochemical features typical of myocardiac layer cells. Such a process would explain the similarities in their staining patterns. We believe that the difference in actin expression detected by GR-ICOR-2 in different sections reflects different stages of heart maturation. This hypothesis is supported by several

studies that have reported the dependence of the spatial distribution of different isoforms of actin on the stage of heart development investigated (Hayward and Schwartz, 1986; Ruzicka and Swartz, 1988; Handel et al., 1989; Sugi and Lugh, 1992). However, the greater expression of actin in cardiac jelly cells may be related to the functional status of these cells (i.e. movement or migration), rather than their stage of differentiation or their origin.

The most markedly positive group of cells were noted in the cushion tissue of the AV canal and in the conal crests, a finding compatible with experimental analyses in different stages of cardiac development (De La Cruz et al., 1972; Argüello et al., 1986).

The dorsal area of positivity in the primitive ventricle is also evidence in support of the observations of Argüello et al. (1986) indicating that the primitive ventricular region of the bulboventricular loop is one of the earliest areas to undergo differentiation, followed by the AV canal.

Observations obtained thus far in our laboratory allow us to characterize GR-ICOR-2 as an IgM type mAb which recognizes sarcomeric actin protein in the cytoplasm of cells in the myocardium as well as in the cardiac jelly.

The mAb GR-ICOR-2 cross-reacts with cardiac and skeletal muscle of the chicken, rabbit and human. The lack of cross-reactivity of this mAb with actin of mouse can be explained by the fact it is unusual for a mAb produced in mouse to react with autoantigens of mouse. On the other hand, the antigenic homology between the different actins may account for the cross-reaction observed with our mAb.

Because of this wide spectrum of interspecific cross-reaction, GR-ICOR-2 may be useful as a highly specific reagent in comparative studies of different processes of differentiation and maturation in cardiac and skeletal muscle.

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