Xenopus as an experimental model for studying evolution of hsp–immune system interactions

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Abstract

The frog Xenopus provides a unique model system for studying the evolutionary conservation of the immunological properties of heat shock proteins (hsps). General methods for maintaining and immunizing isogenetic clones of defined MHC genotypes are presented together with more recently developed protocols for exploring hsp-mediated immune responses in vitro (proliferative and cytotoxic assays) and in vivo (adoptive cell transfer and antibody treatment) in adults and in naturally MHC class I-deficient larvae. Finally, techniques to study modalities of expression of the endoplasmic reticulum resident gp96 at the cell surface of tumor and normal lymphocytes are considered.

Keywords: Xenopus; Proliferative assay; Cytotoxicity; CFSE; Heat shock protein; gp96; NK cells; CD8 T cells; Phylogeny; Evolution

1. Introduction

The South African clawed frog Xenopus has long been used successfully as the ectothermic (cold-blooded) vertebrate species of choice for gaining a better appreciation of the phylogeny of the complex immune system. Not only is a comparative approach for studying evolution of immunity still uncovering im-

presegeny1succ41.8(gp96t)-6.8(549786.55su8(g-79n6.55sue)-axon.6.55surfF488.55surfexJT*[(de(ax)-4/F21Tf3.932903D[(Xenopus

(1) Two developmentally and physiologically distinct immune systems coexist in the same species. The adult immune system is fundamentally similar to that of mammals (e.g., rearranging TCR and Ig genes, MHC class I- and class II-restricted T-cell recognition; reviewed in [6,7]). In contrast, the larval immune system presents some deficiencies such as poor switching from IgM to IgY [8,9], an incomplete skin graft rejection capacity [10,11], and weak anti-tumor defenses [12,13]. In addition, the absence of classical MHC class I antigens and the proteasome subunit LMP7 in most tissue, including the thymus, until metamorphosis [6,14,15], suggests that T-cell education in premetamorphic larvae is likely to occur in the absence of MHC classical class I antigen-presentation.

(2) The availability of genetically identical MHC-defined clones [2,3] and inbred strains [11,16] of Xenopus (Table 1) permits the study of hsp-mediated immunity in a genetically defined setting and o ers

fertilization with normal sperm following the same technique used for LG frogs.

2.2. Immunization

Gp96 and hsp70 are purified from normal liver or tumor tissue following the same protocol used in mice. Briefly, gp96 is purified by 50–70% ammonium sulfate fractionation and concanavalin A-sepharose and DEAE chromatography [42]. Hsp70 is purified by Blue–Sepharose chromatography to remove albumin contaminants and passage through either an ADP–Agarose or ATP–Agarose column (Sigma Chemical, St. Louis, MO), followed by DEAE chromatography [42,43]. Purity is assessed by SDS–PAGE followed by silver staining and Western blotting. Approximately 20–50 μg of purified gp96 and 5–10 μg of hsp70 can be obtained per ml of Xenopus tissue. A 15/0 solid tumor corresponds approximately to a tissue volume of 5–10 ml and liver from a 100 g adult to a volume of 1–2 ml. We have observed that purified Xenopus gp96 tends to degrade rapidly at 4 °C especially after it has been concentrated. This may be due to an endopeptidase activity [44]. To prevent degradation, the DEAE eluate is concentrated by centrifugation (30 kDa filter Sigma Z36, 464-9), aliquoted, and frozen at −70 °C; samples are then used to determine its purity, etc. The reasons for the low yield of purified hsp70 are unclear.

The dermis of postmetamorphic Xenopus skin is thin, devoid of fat tissue, and unattached to the subcutaneous muscle. Therefore, the best route for hsp immunization of adult Xenopus is a s.c. injection in the dorso-posterior region, where there is an active lymphatic drainage to the spleen [45]. Note that the spleen and thymus are the only organized lymphoid tissues in amphibians which lack lymph nodes [9]. In our experience, the s.c. route of injection in adults gives more consistent results than the

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to MSF. This medium is used to culture A6 fibroblasts and to obtain T-cell growth factor (TCGF)-enriched supernatants from PHA-stimulated splenocytes [39,52].

- ASF-A6: ASF supplemented with 20% supernatant from the A6 kidney fibroblast cell line (ATCC: CCL 102).
- ASF-A6-XS: ASF supplemented with 20% A6 supernatant and 0.25% normal Xenopus serum.

2.4.3. Procedure

Animals are euthanized in 1–5% TMS and their ventral skin is washed 2× with 70% ethanol before performing a laparotomy under aseptic conditions. The spleen is placed in a petri dish in ABPS and dissociated between two pieces of nylon mesh. The dissociated cells are collected by 10 min centrifugation at 400g. If needed, erythrocytes can be removed by centrifugation (10 min 400g) on a ficoll cushion (Sigma 1.077) and washed 2× in APBS. E ector cells are resuspended in ASF-A6-XS medium at a final cell density of 5×10^6/ml. Two-year-old adult LG frogs are smaller (100 g) than outbred X. laevis and their spleens contain between only 10 and 20×10^6 lymphocytes. Generally, spleens from several animals need to be pooled.

Splenocytes or tumor stimulators are irradiated (50 Gy), washed 3× in APBS, and resuspended at 2.5×10^6 cells/ml in ASF-A6-XS medium. E ector cells are mixed with stimulators in a 2:1 ratio in 24-well flat-bottom plates (5×10^6 e ector + 2.5×10^6 stimulator/2 ml/well) and cultured for 6 days at 25–27 °C. For the cytotoxic assay, 100 µl/ml of 10× concentrated TCGF-enriched supernatant [39,52] is added.

2.5. CFSE staining

2.5.1. Materials

Five molar CFSE (dissolved in DMSO, aliquoted, and stored at −20 °C) and staining bu er (APBS + 1% FBS and 0.1% NaN_3).

stimulator cells; the surface marker recognized by this mAb is not expressed by splenocyte [23,24]. At this time, the relative contributions of a direct antigen-specific induced proliferation involving MHC-presentation and an indirect non-specific cytokine-mediated proliferation are unknown. Further study, using cell populations sorted by magnetic microbeads (MACS) before in vitro culture should clarify this issue.

2.7. Study of cell-mediated cytotoxicity vitro by the JAM assay

Cell-mediated cytotoxicity by Xenopus T cells [48,49] and NK cells [29] has been characterized in vitro by the classical radiolabeled chromium-release assay. Progress in understanding the biology of apoptosis resulting from the interaction between killer cells and their targets has given rise to questions about the physiological relevance of this assay. The release of radiolabeled chromium in the medium by the dead targets requires the loss of the integrity of the plasma membrane which is a rather late event in induced cell death [53]. In fact, recent evidence indicates that perforin induces plasma membrane damage only at high, non-physiological concentration, whereas it still induces potent apoptosis at low concentrations through a mechanism similar to endosomal internalization of virus [54]. The monitoring of DNA loss by fragmentation (JAM assay) developed by Matzinger [55] for mice appears more appropriate than a chromium release assay for determining cell-mediated killing, since DNA fragmentation (DNA ladder formation) is a more reliable criterion for judging apoptotic death. Details of the protocol, as modified for Xenopus, are presented below with an overview in Fig. 2.

Xenopus 6-day-old LG-15 normal PHA-induced splenic blast targets are labeled for 20 h.; tumor 15/0 targets are labeled for only 2 h (to prevent apoptosis) at 26 °C with 5 mCi/ml [^{3}H]TdR (NEN Life science product Boston MA, NET 027, 6.7 Ci/ml). PHA-lymphoblasts are thoroughly resuspended with a pipettor or a syringe with a 25-gauge needle to disrupt cell aggregates. After washing 3□ in APBS containing 1% BSA, $1 \square 10^4$ radiolabeled 15/0 targets, or $2.5 \square 10^4$ lymphoblasts, are

extent of apoptosis due to thymidine incorporation, the same number of target cells are distributed (in triplicate) in a separate plate at the beginning of the assay and directly harvested. Cells are harvested with a 96-well harvester (Betaplate,Wallac) and thymidine loss is determined by β-scintillation spectrometry. Specific killing is determined as follows: % DNA loss ¼ $T \square \eth T$ þ $E\eth/T \square$ 100, where T is the incorporated label (cpm) in targets after 4 h culture without e ectors, E is the experimentally retained DNA in the presence of killers. The accuracy of the assay greatly depends on targets that must be in an active proliferative phase for good thymidine incorporation and minimal apoptosis induction. In our hands, the maximum incorporation by spleen cells from LG animals was obtained after 6 days of culture with 0.5 μg/ml of PHA. Cell death is usually minimal (less than 20%) and does not require removal of dead cells by ficoll separation. Tumor cells should be fed with fresh medium 1–2 days before the test and their density should not exceed $1 \square 10^6$/ml (< 5% death) at the time of the thymidine pulse.

As in mice, our results indicate that the JAM assay is more sensitive than the classical ^{51}Cr release assay in Xenopus (Fig. 3), and it allows to use as few as 10,000 to 20,000 targets in contrast to 50,000 needed for the chromium release assay [2]. In addition to our improvement of the in vitro stimulation conditions by supplementing the medium with normal Xenopus serum and homologous ''IL-2''-enriched supernatant (see the proliferative assay section), the specificity of the JAM assay described in this section is further increased by sorting stimulated e ector cells with antibody-coated magnetic microbeads (MACS).

2.8. Cell purification or depletion by magnetic bead cell sorting

2.8.1. Materials

Anti-mouse IgG or IgM-conjugated magnetic microbeads (MACS, Miltenyi Biotec), MACS MS column (130-042-201). MACS bu er (APBS with 0.5%BSA and 4 mM EDTA).

2.8.2. Procedure

Spleen cells that have been stimulated in vitro are incubated with either anti-CD8 AM22 (IgM isotype) or anti-NK cell 1F8 (IgG1 isotype) mAbs, and antibody-coated cells are positively selected using MACS coupled with mouse-specific anti-μ chain or anti-IgG following the manufacturer's instructions. Between 10 and $20 \square 10^6$ cells are incubated for 30–60 min on ice with anti-CD8 mAb (200 μl AM22, 200 μl APBS + 1% BSA, and 100 μl sterile distilled water). Cells are washed 2□ with APBS + 1% BSA and incubated 15–20 min at 4 °C with rat anti-mouse IgM MACS (80 μl, MACS bu er and 20 μl of MACS). Cells are washed 1□ and resuspended in 500 μl of MACS bu er. Cell subsets are separated using a MACS-MS column following the manufacturer's protocol. Both positive and negative populations are put back into culture (ASF-A6-XS medium) overnight at 27 °C. NK cells are sorted according to the same protocol using 1F8 mAb supernatant and anti-mouse IgG conjugated MACS. The purity of the cell sorting is controlled by flow cytometry or if there are few cells, by, fluorescence microscopy, using a fluorochrome-conjugated secondary Ab; 90–95% purity is currently obtained.

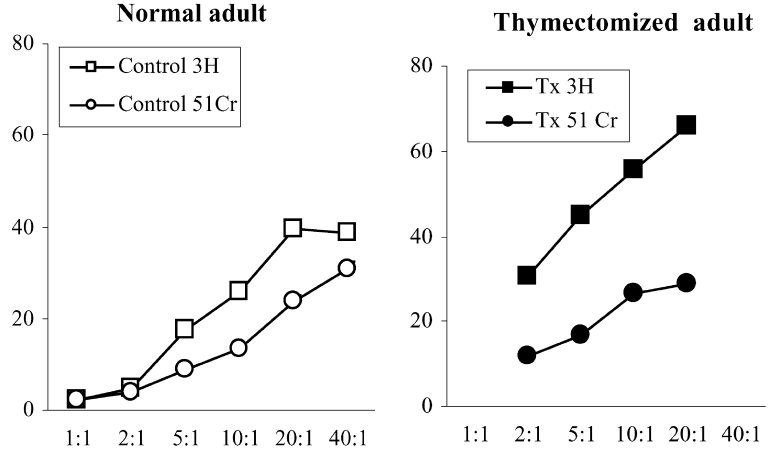

Fig. 3. Comparison between the JAM and ^{51}Cr release assay. Splenocytes from 3- to 4-month-old outbred young frogs that were either thymectomized at an early larval stage or normal, cultured for 48 h in Con A supernatant [29], and tested for NK activity against MHC-negative lymphoid tumor B3B7 targets. One million B3B7 targets were either labeled overnight with 100 μCi ^{51}Cr, or 3 h with 5 μCi/ml ^{3}HTdR. 50,000 ^{51}Cr-labeled targets and only 10,000 ^{3}HTdR targets were used per well in 5 h assays at 26 °C. Variation within triplicate groups was less than 10% of group mean cpm.

3. Studying Xenopus HSP-mediated immune response in vivo

The availability of Xenopus MHC-defined inbred stains and clones provides us with a unique opportunity (for ectothermic vertebrates) to study in vivo the fate of lymphocyte subsets by adoptive transfer both in adults

CFSE^{b} B cells are found in similar number. Note also that both CD5^{b} and CD8^{b} anti-tumor primed T-cells have proliferated, as indicated by the decreased CFSE signal of a substantial fraction of the cells. In addition, more primed CFSE^{b} T-cells than naïve T-cells can be found in the host tumor (i.e., tumor-infiltrating lymphocytes or TIL; Fig. 4).

3.2. Impairment of e ector function by antibody treatment

Another way of studying immune e ectors in vivo that has been recently developed in our laboratory makes use of neutralizing antibody treatment protocol. Specifically, we have shown that CD8 cells can be depleted for more than a week following the injection of the anti-CD8 mAb AM22 [20]. More recently, we have also successfully used the anti-NK mAb 1F8 to impair alloimmunity and tumor immunity; but unlike the anti-CD8 mAb, this anti-NK cell reagent does not deplete 1F8-expressing cells [58]. As described below, mAb treatment can be performed both in adults and tadpoles.

3.2.1. Adults

All mAbs used for in vivo treatment are from ascites fluid produced in BALB/c mice that had been pre-treated with Freund's complete adjuvant (0.5 ml/mouse i.p.) and injected one week later with the hybridomas (1×10^6 cells/0.5 ml/mouse). Ascites is diluted 10× in APBS (approximately 1 mg/ml of protein) and sterilized by filtration through a 0.2 μm filter.

Unanesthetized adult frogs are injected i.p. (25G 5/8 needle) on their ventral surface just above the thigh with 0.2 ml (100 μg proteins) of diluted mAb 1 day prior to tumor challenge. Treated animals are kept in clean water containing a fungicide (50 μl/10 L, Aquarium Products, Glen Burnie, MD). In case of excessive mortality, penicillin plus streptomycin (0.005% final in water, Sigma) can be added. We have used this approach recently to begin an in vivo characterization of e ector cells involved in hsp-mediated anti-tumor responses. In a preliminary experiment (Fig. 5A), groups of 5 LG-15 adults were immunized twice with gp96 purified either from 15/0 tumor. One day before tumor challenge frogs were injected either with APBS (vehicle), anti-CD8 or anti-NK mAbs. An additional, unimmunized group was also injected with APBS. The delay in tumor appearance obtained by immunization was abrogated in animals pre-treated with either anti-CD8 or anti-NK mAbs (Fig. 5A).

3.2.2. Tadpoles

Tadpoles at pre-metamorphic stage 55–56 (≈1 month post fertilization, 37) are injected with 5–10 μl i.p. on their ventral side just above the intestinal area using a pulled Pasteur pipette attached to rubber tubing. Tadpoles are more fragile than adults and need to be anesthetized in a solution of 0.1 g/L of TMS. Typically, tadpoles must be immunized twice (2 week interval) with gp96 before mAb treatment and tumor transplantation. During this time, some of the tadpoles may start metamorphosing. It is useful, therefore, to prevent metamorphosis by adding sodium perchlorate (1 g/L) to their aquarium water [59]. This goitrogen e ectively competes with iodine that is essential for the biosynthesis of thyroid hormone. Although this treatment at early larval stages temporally delays (rather than completely arrests) the expression of MHC class I molecule by a few weeks [60], it permanently blocks most of the drastic morphological and physiological transformations that occur at di erent rates in a population of outbred or even cloned tadpoles. As such, the use of perchlorate considerably simplifies the analysis of data. Between 10 and 14 days after tumor transplantation, peritoneal fluid is

concern when studying the modality of gp96 surface expression is to eliminate artifacts of dead cells or cells with damaged plasma membranes. We describe below a technique, originally published by Wiest et al. [61], that not only allows one to rule out such artifacts but also constitutes a good way of further investigating the mechanisms involved in the gp96 surface expression (e.g., turnover, specificity).

4.1. Cell surface labeling and immunoprecipitation

4.1.1. Materials

Mouse mAb specific for the KDEL c-terminal ER retention signal [10C3; StressGen (Biotechnologies, Victoria, BC, Canada)] and rat mAb specific for gp96 [clone 9G10; Neomarkers (Fremont, CA SPA-850)].

4.1.2. Procedure

Procedures for cell surface biotinylation, lysis in NP-40, and immunoprecipitation with protein G have been detailed elsewhere [19]. Before and after labeling, cells

and clones together with a well developed set of reagents and methodologies (some of which have been described in this review), make Xenopus an important non-mammalian system for exploring this interface. We hope that the information and techniques provided here will encourage investigators to recognize the value of, and more fully exploit, this model.

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Appendix A. Reagents

- Iscove's Modified Dulbecco's Medium (Gibco-BRL cat. # 12200-036).
- Non-Essential Amino Acids Solution 100□ (Gibco-BRL cat. # 12383-014).
- Insulin from Bovine Pancreas (Sigma I-6634) dissolved at 5 mg/ml in water and aliquoted at −20 °C.
- 2-Mercaptoethanol 55 mM (Gibco-BRL cat. # 21985-023).
- Primatone Enzymatic digest of animal tissue (Sheffield Products Division) dissolved 10% in water.
- Kanamycin solution (10 mg/ml; Sigma K0129).
- Penicillin–Streptomycin (10,000 U/ml; Gibco-BRL cat. # 15140-122).
- Fetal Bovine Serum (Atlanta biologicals cat. # S11150) Heat inactivated at 56 °C for 30 min, then aliquoted in 10 ml/tube, and stored at −20 °C.
- Phytohemagglutinin-P (PHA).
- Bovine Serum Albumin (Sigma A-4503) BSA.
- A6 kidney fibroblast cell line (ATCC: CCL 102).

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