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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 important new information, but *Xenopus laevis* is also proving to be an excellent model system for studying fundamental immunological questions that are not phylogenetically restricted to a given taxon. For example *Xenopus* is a unique nonmammalian model for studying both the basic biology and evolution of heat shock proteins (hsps)[1]–immune system interactions. Given the high degree of phylogenetic conservation of the structure of hsps, the involvement of these proteins in immune responses is likely to be evolutionarily conserved. Indeed, we have shown that the capacity of gp96 and hsp70 to chaperone antigenic peptides, to elicit potent specific cellular adaptive immune responses, and to interact with antigen-presenting cells, is common to amphibians and mammals [1,2]. Considering that one would have to go backward in time more than 350 million years to find an ancestor common to the genus *Xenopus* and mammals [3,4], our data clearly provide strong evolutionary validation for a critical role of hsps in immune responses. Furthermore, additional immunologically related properties found in *Xenopus* suggest that certain hsps may be ancestral agents of immune surveillance that played an important selective role in the evolution of the vertebrate immune system. For example: *Xenopus* gp96 is actively expressed at the surface of a subset of IgM^+ B-cells [5]; both *Xenopus* hsp70 and gp96 elicit potent peptide-specific anti-tumor immunity against MHC class I-negative tumors [1]; and *Xenopus* gp96 generates an innate type of anti-tumor response that is independent of chaperoned peptides in naturally MHC class I-deficient tadpoles [2].

The unique features that make *Xenopus* an attractive model for studying phylogenetic aspects of both immunity in general and hsp–immune system interactions in particular, include:

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[1] *Abbreviations used:* APBS, amphibian phosphate buffered saline; hsp, heat shock protein; MHC, major histocompatibility complex; mAb, monoclonal antibody.

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(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 offers the opportunity to adoptively transfer cells without accompanying histoincompatibilities [17]. MHC-restricted cytotoxicity against major and minor histocompatibility (H) antigens has been studied in *Xenopus* and we have recently shown that such MHC-specific cytotoxicity involves $CD8^+$ T-cells [2].
(3) Well-characterized *Xenopus* lymphoid tumor cell lines (Table 2) have been developed from spontaneously arising thymic lymphoid tumors [12,13,18,19]. Importantly, some of these lymphoid cell lines do not express MHC class I and class II mRNA or protein [12,15,20] and have maintained their tumorigenicity when transplanted in histocompatible hosts [12,13].
(4) A relatively large panel of *Xenopus*-specific mAbs and molecular reagents is available for dissecting the cellular basis of hsp-associated immune responses (Table 3).

In addition to providing a unique non-mammalian system to study fundamental immunology and its evolution, *Xenopus* has recently become a key model for understanding putative defects in the immune defense against viral and fungal pathogens that have been causally implicated in the world-wide declines in amphibian populations and species [34,35]. From a practical perspective, it is not unreasonable to speculate that our understanding hsp–immune system interactions in *Xenopus* could eventuate in hsp-based vaccination protocols for endangered species in zoos, if not in the wild.

General information about raising *Xenopus* in the laboratory [36,37] and several methodologies to study immunity in *Xenopus* [19,37–40] are detailed elsewhere. This review will focus on recent techniques that have been developed to study hsp-mediated immune response in vitro and in vivo.

Table 1
Xenopus MHC-defined strains and clones

	Name, (MHC genotype)	Ref.
Partially inbred, MHC homozygous strains	F, J, R, K, G	[11,16]
Isogenetic laevis/gilli (LG) clones with identical heterozygous (*a/c*) MHC genotype but different minor H genotype	LG-6, 7, 15, 46	[2,3]
MHC-disparate LG isogenetic clones	LG-3 (b/d), LG-5 (b/c)	[2,3]
Isogenetic laevis/mulleri (LM) clones	LM3	[2,3]

Table 2
Characteristics of four different *Xenopus* cloned thymic tumor cell lines

Name of tumor cell line	BB7	ff-2	15/0	15/40
Genetic background of initial tumor-bearing host	Partially inbred MHC homozygous F strain		LG-15 (MHC *a/c*) isogenetic clone	
T-cell surface markers (CD8, CD5, pan T-cell)	+++	+++	+++	+++
Ig mRNA heavy chain	–	+	+	+
Light chain	–	+	+	+
Ig protein	–	–	–	–
MHC class I mRNA and protein	–	+	–	+
MHC class II mRNA and protein	–	–	–	+
Surface gp96	+++	++	+	++
Tumorigenicity in syngeneic				
Larvae	–	+	++	+
Adult	–	–	++	++

Table 3
Expression pattern of *Xenopus* lymphocyte surface markers detectable with currently available mAbs

Xenopus markers (mAbs)[a]	Expression pattern	Ref.
CD8 (AM22, F17)	Larval and adult thymocytes (70–80%) and T-cells (about 20% of splenocytes). All lymphoid tumor lines	[21,22]
CTX (X71, 1S9.2)	Larval and adult thymocytes (60–70%); no consistent expression in peripheral lymphocytes. All lymphoid tumor lines, gut epithelial tissue	[23,24]
XT1 (XT1)	Most, but not all, larval and adult T-cells; earliest marker of thymocytes. All lymphoid tumor lines	[25]
MHC class I (TB17)	Ubiquitous in adult; all lymphopoietic lineages. Not expressed until metamorphosis	[26]
MHC class II (AM20, 14A2)	Thymocytes, B and T-cells (99% of spleen lymphocytes), only B-cells in larvae	[21]
CD5 (2B1)	Thymocytes (> 95%), T-cells and some PMA-activated IgM^+ B cells. All lymphoid tumor lines	[27]
CD45 (CL21)	T and B cells. All lymphoid tumor lines	[28]
NK-like (1F8)	Non-B and non-T, peripheral lymphoid cells	[29]
Anti-human CD3 epsilon (CD3-2)	Cross-reacts with *Xenopus* CD3 epsilon, and coprecipitates the TCR-CD3 complex of T-cells and lymphoid tumor lines.	[30]
RC47	Leukocyte lineage from very early stage. Thymic cortex and medulla (> 90% of total thymocytes)	[31]
IgM (10A9)	Larval and adult B cells	[32]
IgY (11D5)	Some larval and adult B cells	[32]
IgX (410D9)	Some larval and adult B cells, especially in the gut	[33]

[a] No mAbs specific for CD4 or TCR have been described so far.

2. Studying *Xenopus* HSP-mediated immune responses in vitro

2.1. Maintaining MHC-defined Xenopus inbred strains and isogenetic clones

2.1.1. Materials

UV light source at 253.7 nm (Gelman Intrument, NY), human chorionic gonadotrophin (HCG Sigma CG5), De Boer's solution (110 mM NaCl, 1.3 mM KCl, and 0.4 mM $CaCl_2$), and DNA staining buffer (1% Na-citrate, 0.1% Triton X100, 100 mM EDTA, 1 mM PMSF, and 10 mg/ml propidium iodide).

2.1.2. Procedure (modified from 3)

Our laboratory maintains a collection of inbred strains of *X. laevis* and MHC-defined syngeneic clones produced gynogenetically from *X. laevis* × *X. gilli* (LG) hybrids [3]. Several of these clones (LG-6, LG-7, LG-15, LG-17, and LG-46) share the same *a/c* MHC haplotype but have different minor H-loci; others differ from these clones by one (LG-5) or two (LG3) MHC haplotypes (Table 1). These clones and inbred strains permit classic adoptive transfer and transplantation protocols to be executed as they are routinely done in mice.

To produce clonal progeny, ovulation of LG females is induced by an initial subcutaneous (s.c) injection of 10 IU (for ~20 g animals) to 100 (for ~200 g animals) of HCG followed by a second injection of the same amount the following day. Several hours later (4–6 h at 22–23 °C when the cloaca is red), eggs are mechanically expressed into a petri dish where they are parthenogenetically activated with UV irradiated sperm (7 min at 15 cm from a at 253.7 nm UV source) from an outbred male. To obtain sperm, testes from an outbred animal are macerated in De Boer's saline solution. The tonicity of this solution inhibits sperm mobility and sperm in De Boers solution remain viable for several days at 4 °C. Motility is restored by adding dechlorinated water (decreasing the tonicity). Sperm viability/activity after UV irradiation (and addition of water) can be confirmed by phase contrast microscopy. After adding sperm to the eggs and waiting a few minutes until the spermatozoa adsorb to the egg vitelline membrane, a small volume of dechlorinated water is added to the petri dish for 1 or 2 min followed by flooding the dish with water. Successful fertilization is noted by the rotation of the eggs so that the black animal pole of the egg is dorsal. Fertilized eggs are left overnight in a large volume of water. The following morning, large diploid eggs are separated from the smaller aneuploid ones. If spermatozoa are not properly inactivated, triploid eggs will be produced and the resulting progeny will not be clonal. The efficacy of UV inactivation should be checked periodically by fertilizing haploid eggs from a normal diploid female with putatively irradiated sperm. If inactivation has been successful, the resulting embryos will be haploid and will not develop further than the tailbud stage (i.e., haploid syndrome). The ploidy of adult LG animals can also be tested by flow cytometry [41]. To this end, anesthetized frogs are bled from the dorsal tarsus vein in the foot [38] and blood is collected in APBS containing 10 U/ml of heparin. After 2 washes in APBS, blood cells are stained overnight (10^6 cell/ml) in DNA-staining buffer and the stained nuclei are then analyzed by flow cytometry on a linear scale. Normal diploid and triploid controls are used as a reference. Inbred J [16] and F [11] stains are usually maintained by in vitro

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 reproducible results than an i.p. injection. Ten micrograms of purified hsp (gp96 or hsp70) diluted in 0.3 ml of APBS is injected (1 ml syringe and 27-gauge needle) two to three times at a two week interval. Animals are used for experiments 2 weeks after the last injection.

Frogs can be immunized against major or minor H antigens by multiple skin grafts [2,14]. Grafting is done according to published methods [46] by inserting a piece of ventral skin (5 mm^2) under the dorsal skin of a recipient and removing the overlying host skin 24–48 h later. The onset of rejection is marked by initiation of pigment cell death and rejection is considered complete when all pigment cells in the graft are destroyed. Animals are generally boosted by a second-set graft.

2.3. Assessment of proliferative responses in vitro using CFSE labeling

Immunization with peptide-bearing gp96, like immunization with other antigens, leads to the priming of effector cells that should become readily activated upon subsequent exposure (secondary response) to the chaperoned peptides. Early events in such immune recognition include proliferation and expansion of effector cell subsets. Proliferative responses in vitro have been studied in *Xenopus* mainly by the mixed lymphocyte reaction (MLR) using a standard tritiated thymidine incorporation method and MHC-disparate splenocytes [38]. This technique was responsible for the first identification of MHC in any ectothermic vertebrate [47]. It has provided a useful tool to study the generation of cytotoxic T cells [48,49], diversity of *Xenopus* MHC haplotypes [48], and the critical involvement of MHC class-II in antigen-specific proliferation [50]. However, the MLR provides only indirect information in that it does not allow one to distinguish which particular cells or cell populations are actually proliferating. This is especially problematic in *Xenopus* where recombinant cytokines and antigen presenting cell lines are not available, and MHC-restricted CTL epitopes are not defined. To overcome these difficulties, we have explored another approach that takes advantage of the fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CSFE) that binds convalently to diverse cellular proteins without associated toxicity or marked functional effects [51]. The initial fluorescence intensity of $CFSE^+$ cells decreases by half at each cell division. It is possible to follow the response of pre-labeled $CFSE^+$ effector subsets using mAb against different surface markers and multiparametric flow cytometry. The method described below has been designed to characterize (i.e., specificity, kinetics, and magnitude) the proliferative response of different hsp-primed *Xenopus* lymphocyte cell populations to various stimulatory conditions.

2.4. In vitro stimulation

2.4.1. Materials (suppliers for reagents mentioned in this section are found in the Appendix A)

- 5% tricaine methanesulfonate (TMS)
- Amphibian phosphate buffered saline (APBS): 6.6 g/L NaCl, 1.15 g/L Na_2HPO_4, and 0.2 g/L KH_2PO. Adjust pH to 7.5 with 10 N NaOH and sterile filter through 0.2 μm filter.

2.4.2. Media (modified from 19)

- Mammalian serum free basic medium (MSF): mix one package of powdered Iscove DMEM basal medium; 10 ml insulin, 10 ml non-essential amino acids, 10 ml penicillin–streptomycin; 3 ml of primatone (Sheffield Products Division), 1 ml of 2-mercaptoethanol, and 3.02 g $NaHCO_3$ in water. Adjust pH to 7.0 with 10 N NaOH and final volume to 1 L. Filter through 0.2 μm filter and store at 4 °C.
- Amphibian SF medium (ASF): Add 30% of double distilled water, 5% FBS, and 10 μg/ml of Kanamycin

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 400*g*. If needed, erythrocytes can be removed by centrifugation (10 min 400*g*) on a ficoll cushion (Sigma 1.077) and washed 2× in APBS. Effector 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. Effector cells are mixed with stimulators in a 2:1 ratio in 24-well flat-bottom plates (5×10^6 effector + 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 buffer (APBS + 1% FBS and 0.1% NaN_3).

2.5.2. Procedure

Aliquots of 5M CFSE are thawed and diluted 1:100 in APBS (50 mM final concentration). Splenocytes from immunized donors are collected as previously described and suspended in APBS at $2–5 \times 10^7$ in 450 μl of APBS in a 15 ml conical tube and stained for 15 min in the dark at 27 °C by adding 50 μl of 50 mM CFSE (5 mM final concentration). Following incubation, this suspension is brought to 15 ml with cold APBS and centrifuged at 400*g* for 10 min. The cells are resuspended by repeated pipetting in 10 ml of cold APBS, counted, and centrifuged as above. The resulting pellet is resuspended and the cell number is adjusted to 5×10^6 cells/ml in ASF medium. CFSE-stained cells are then mixed with irradiated unstained 15/0 tumor stimulators or, as a control, with normal naïve syngeneic splenocytes. Additionally, a control well of $CFSE^+$ splenocytes without stimulators is plated to give a baseline CFSE fluorescence level for unstimulated cells. Cells are collected after 4–6 days in culture, washed in APBS, and resuspended in cold staining buffer to an approximate concentration of $1–5 \times 10^7$ cells/ml to prepare them for antibody staining and flow cytometry.

2.6. Two-color flow cytometry

From 0.5 to 1×10^6 cells in a volume of 25 μl are added per well of a V-bottom 96-well plate. Cells are first stained with various *Xenopus*-specific primary mAbs listed in Table 3. Two hundred microliters of undiluted hybridoma supernatant or 1 μg/ml of purified *Xenopus* specific mAbs is added to each well and the plates are incubated on ice for 1 h. Mouse isotype-matched mAb controls are also included. Plates are centrifuged at 4 °C for 5 min, and the supernatants are discarded by briskly inverting the plate and blotting on a paper towel. The plates are then washed by adding 200 μl of cold staining buffer rapidly enough to resuspend the cells, centrifuged, and then washed a second time. Cells are then stained with a phycoerythrin (PE) or allophycocyanin (APC)-conjugated goat anti-mouse (Fab2) secondary antibody diluted in staining buffer. To reduce non-specific staining, secondary Abs are adsorbed 1:1 by volume 2× 30 min on *Xenopus* erythrocytes. Some anti-*Xenopus* mAbs are conjugated directly to PE-fluorochrome (2B1) or biotin (AM22, F17), the latter being detected by PE- or APC-conjugated streptavidin (Pharmingen, San Diego, CA). Since CFSE fluoresces at the same wave length as FITC, the secondary antibody must be tagged with a different fluorochrome (i.e., PE or APC). All incubations are carried out for 30 min on ice followed by two washes. After the final staining, the cells are resuspended, transferred to flow tubes (LPS #1.284502), adjusted to a cell density of $2–5 \times 10^5$ cells/ml, and analyzed by flow cytometry on a FACSCalibur (Beckton Dickinson); 20–50,000 total events are collected.

Typical results using the experimental protocol described here are presented in Fig. 1. Note that T-cells from animals that were immunized with 15/0 tumor-derived gp96 respond by proliferating upon exposure to 15/0 stimulator cells, and at least three rounds of cell divisions (three peaks) can be detected. This response is greater than the proliferation seen when cells from an immunized frog are mixed with cognate stimulators. Proliferation of T cells is greater than the response of other cells but when other cell types are dividing.

After 5 days of culture, stimulator cells have been reduced to cell debris or apoptotic bodies that are easily gated out by side and forward scatter. Alternatively, the mAb X71 can be used to detect residual 15/0 tumor

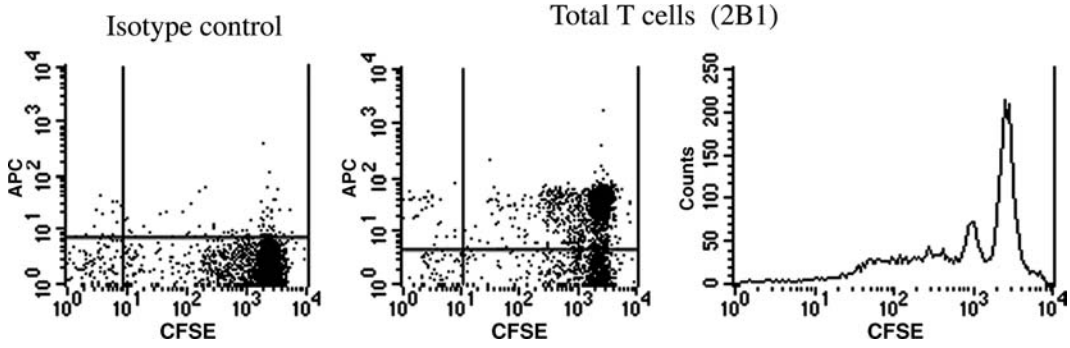

Fig. 1. In vitro proliferative assay using CFSE staining. In vitro proliferation of CFSE-labeled LG-6 splenocytes from 15/0-gp96 immunized frogs stimulated for 5 days with irradiated 15/0 tumor cells. Cells were stained with anti-CD5 mAb (2B1) or isotype control followed by APC-conjugated goat anti-mouse (Fab)2 secondary Ab. $CD5^+$ cells were gated and analyzed for CFSE staining intensity (Histogram). Note that T cells have undergone at least three cell divisions (three peaks).

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, 1×10^4 radiolabeled 15/0 targets, or 2.5×10^4 lymphoblasts, are distributed into wells of a 96-well plate (in triplicate) and incubated for 4 h at various effector:target (E:T) cell ratios (i.e., 20:1, 10:1, 5:1, and 1:1). To determine the

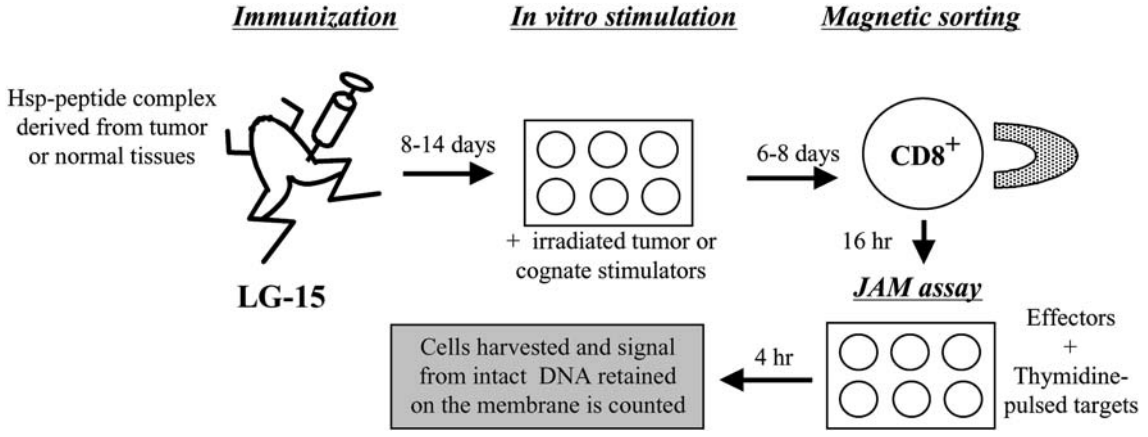

Fig. 2. General protocol to characterize in vitro $CD8^+$ killing activity.

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 $= T - (T + E)/T \times 100$, where T is the incorporated label (cpm) in targets after 4 h culture without effectors, 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 1×10^6/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 effector 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 buffer (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 20×10^6 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 buffer and 20 μl of MACS). Cells are washed 1× and resuspended in 500 μl of MACS buffer. 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.

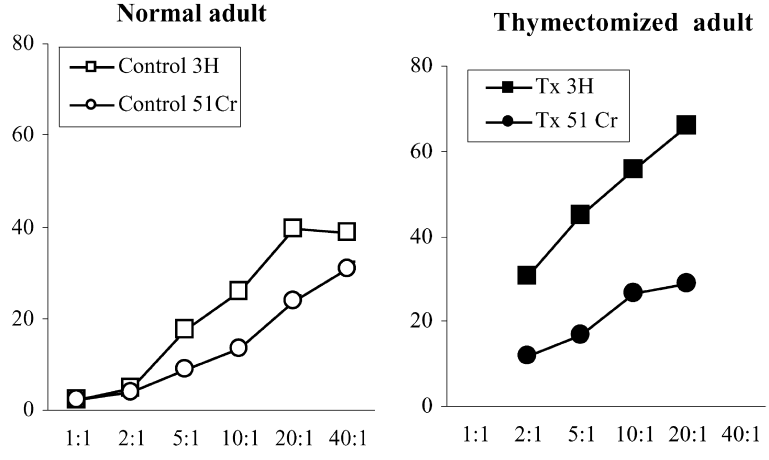

Fig. 3. Comparison between the JAM and ^{51}Cr release assay. Splenocytes from 3- to 4-month-old outbred young frogs that were either thymectomized at an early larval stage or normal, cultured for 48 h in Con A supernatant [29], and tested for NK activity against MHC-negative lymphoid tumor B3B7 targets. 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 and naturally class I-deficient tadpoles. This approach is complemented by the possibility of neutralizing effector function by antibody treatment [20]. Splenocytes can be readily transferred between isogenetic (cloned) animals, with no resulting immune response by either the host or the donor cells [41]. This phenomenon can be exploited to study antigen-specific proliferation of immune cells in vivo.

3.1. Adoptive cell transfer

Adoptive cell transfer in *Xenopus* has been used to study allotolerance during metamorphosis [46] and hematopoiesis, using polyploidy as a genetic marker to follow the transferred cells [41,56,57]. Adoptive transfer of polyploid donor cells provides a good way of identifying cells that have differentiated from progenitors during embryogenesis, However, this technique is less suited for studying the rapid expansion of antigen-specific lymphocyte effectors during a secondary immune response, because it is not possible to determine whether a larger number of effectors results from a more efficient transfer, a difference in homing, or from real cell cycling. CFSE labeling, now being used in mammals [51] and for in vitro proliferative studies in *Xenopus* (see previous section), is a powerful alternative technique for following cell division in vivo. This technique, adapted for use with *Xenopus*, is described below.

LG-15 adult frogs are immunized, as described above, with 15/0 tumor-derived gp96. At the time of the initial immunizations of frogs that will be cell donors, recipients are injected with 2×10^5 15/0 tumor cells suspended in 200 μl APBS. The tumor cells should be injected s.c. to one side of the dorsal midline, on the dorsal surface. Injected 15/0 cell suspensions form solid tumors in LG-15 frogs, the clone from which they were derived, in approximately 4 weeks. Immunized frogs are sacrificed 2 weeks after the second booster injection of 15/0-gp96 and their splenocytes are collected and stained with CFSE as above. Following incubation with CFSE and 3 washes in APBS, the donor splenocytes are suspended in APBS and injected i.p. into recipients that have small palpable tumors. If multiple donors and recipients are being used in this adoptive cell transfer, the cells will be resuspended such that all animals receive the same number of cells in 200 μl.

The tumor-bearing recipients are sacrificed several days after transfer and their splenocytes are collected and analyzed by flow cytometry as outlined above. Approximately 1–2% of the lymphocytes in the spleen will be $CFSE^+$. Differences in proliferative rates of lymphocytes that were previously primed to respond to 15/0 tumor cells and those that were not, are apparent 3 days after adoptive transfer and become most obvious at five and nine days post-transfer (Fig. 4). In this experiment, more anti-tumor primed $CFSE^+$ T cells can be detected in the recipient spleens at day 9, especially $CD8^+$ T cells (4× more than the transferred naïve $CD8^+$ T cells), whereas both transferred naïve and primed

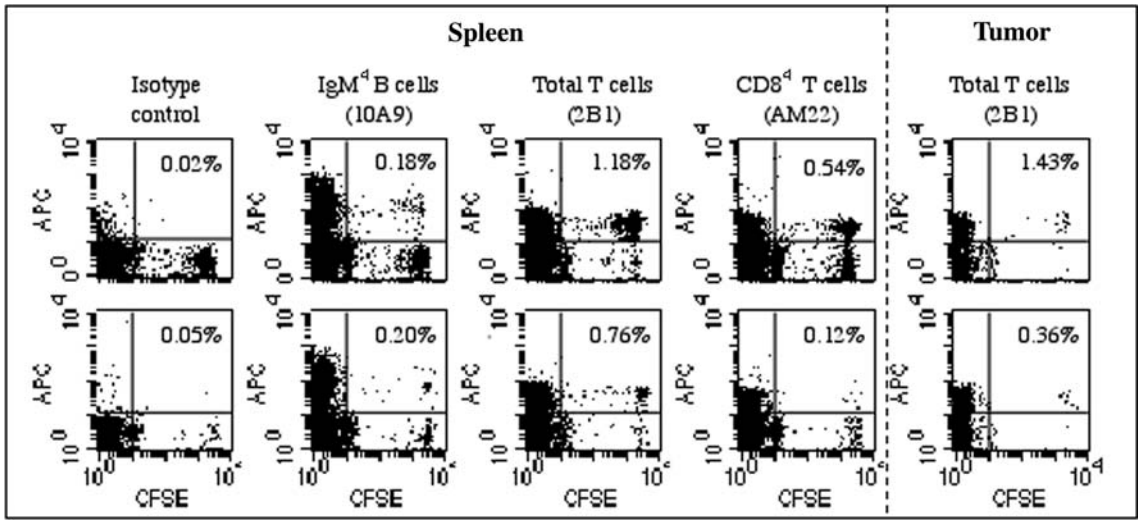

Fig. 4. Adoptive cell transfer. Adoptively transferred $CFSE^+$ splenocytes in the spleen and tumor infiltrate. CFSE-stained (5 nM) splenocytes from either a naïve LG-15 donor (bottom panels) or a LG-15 donor immunized twice with 15/0-derived gp96 (top panels) were transferred i.p. (5×10^6 cells) into a LG-15 recipient bearing a small tumor. Spleen and tumor were collected 9 days later and analyzed by two-color flow cytometry using isotype control, anti-IgM (10A9), anti-CD5 (2B1), or anti-CD8 (AM22) mAbs followed by APC-conjugated goat anti-mouse F(ab)2 secondary Ab. 200,000 events were collected and analyzed on a FACScalibur.

$CFSE^+$ B cells are found in similar number. Note also that both $CD5^+$ and $CD8^+$ 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 $CFSE^+$ 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 effector function by antibody treatment

Another way of studying immune effectors 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 $10\times$ 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 effector 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 effectively 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 different 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 collected separately from each tadpole and added to a 96-well V bottom plate. The volume of each sample is measured with a pipettor and diluted with an equal volume of APBS. Tumor cells are counted using a hemacytometer. Large tumor cells are easily distinguishable from the smaller leukocytes. Alternatively, cells can be first stained with a mAb against CTX (X71), a molecule that, in the lymphocyte lineage, is expressed only by lymphoid tumors or immature thymocytes [23,24], followed by a FITC-conjugated goat anti-mouse (Fab)2 secondary antibody and analyzed by fluorescence microscopy.

Recent evidence (Horton pers, comm) suggests that some NK cells become detectable in the spleen, both by flow cytometry and by the JAM assay, at a late pre-metamorphic stage (st 57–58). At this stage, a fraction of splenocytes begins to express surface class I molecule [60]. Extensive study of cell-mediated cytotoxicity in vitro is hampered by the small number of lymphocytes that can be obtained from these small larvae. Fig. 5B illustrates an attempt to assess, by mAb treatment, the involvement of NK in larval anti-tumor defense. As in adults resistance of larvae to tumor growth is partially impaired by pre-treatment with anti-NK cell mAb.

4. Studying cell surface expression of gp96

Xenopus has been instrumental in showing that cell surface expression of gp96, a resident protein of the endoplasmic reticulum, is not an artifact of dysregulation or in vitro culture conditions, but is a general biological phenomenon [5]. Indeed, we have shown that gp96 is actively directed to, and retained on, the plasma membrane of population of *Xenopus* IgM^+ B cells. Similar gp96 surface expression by immune cells has been observed in bony fish, hagfish (a member of the only vertebrate taxon lacking an adaptive immune system), and the sea urchin (unpublished data). The main

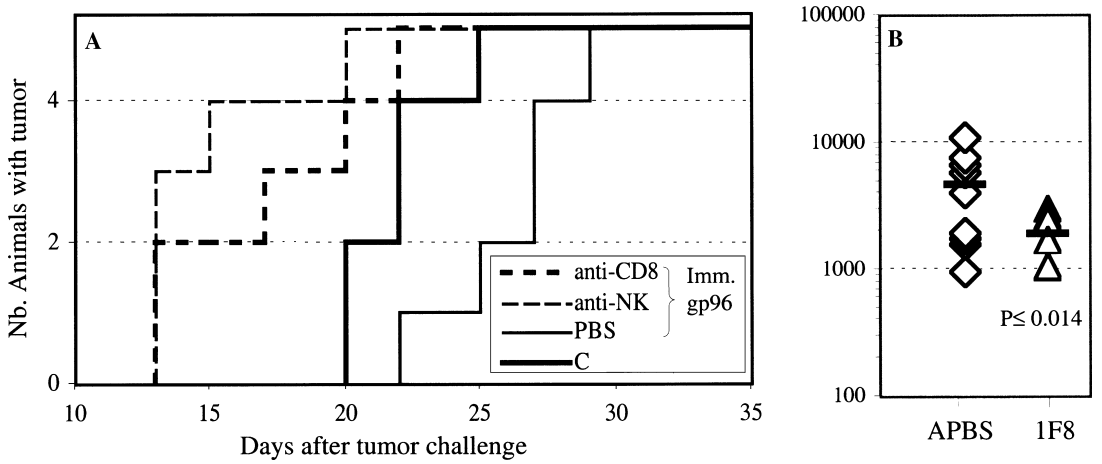

Fig. 5. In vivo mAb treatment. (A) Effect of mAb treatment on hsp-mediated anti-tumor immunity. LG-15 (5 frogs/group) immunized twice with 10 μg of 15/0 tumor-derived gp96 or naïve (C) were injected i.p. with 200 μl of APBS, 10× diluted anti-CD8 (AM22) or anti-NK (1F8) ascites 1 day before challenge with 200,000 live 15/0 s.c. Time for first appearance of solid tumor was monitored. (B) Effect of anti-NK or anti-CD8 mAb treatment on larval immune response against transplanted tumor. Blocked LG-15 pre-metamorphic (st 55) tadpoles (10/group) were injected with 5 μl APBS, or anti-NK (1F8) mAb, and then challenged i.p. 1 day later with 5000 15/0 tumor cells. Tumor cells from peritoneal fluids were counted 7 days later. The horizontal bars represent the means ($p \leqslant 0.014$ by Student's t test for the group treated with anti-NK mAb versus control).

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 are extensively washed three times in APBS that contains 1% BSA. Cell death, determined before lysis by trypan blue dye exclusion, must not exceed 5%. Biotinylated cell-surface lysates (corresponding to $\sim 5 \times 10^7$ cells) are pre-incubated for 1 h at 4 °C with 30 μl/ml of protein G. Hundred microliters of such pre-cleared lysates (corresponding to $\sim 5 \times 10^6$ cells) is incubated overnight at 4 °C with either 100 μl of mAb supernatant and 30 μl protein G, or with a mixture of 3 μl anti-gp96 mAb, 3 μl rabbit anti-rat antibody (Sigma, MO), and 30 μl protein G. Immunoprecipitates are separated on 7.5% SDS–PAGE gels under reducing conditions and transferred onto polyvinyldenefluoride (PVDF) membranes (BioRad, Hercules, CA). Biotinylated proteins are revealed using HRP-conjugated streptavidin and chemiluminescence reagents from Amersham (Arlington, IL). Non-biotinylated proteins are detected after reprobing the membrane with specific antibody followed with a secondary rabbit anti-rat HRP-conjugated antibody.

4.2. Cell surface re-expression assay

4.2.1. Materials

Pronase (Sigma MO P-6911) and brefeldin A (BFA; Sigma).

4.2.2. Procedure

Cells are incubated with pronase (0.4 mg/ml final concentration) for 45 min at 26 °C with occasional agitation and digestion is quenched 10 min on ice with 2.5% BSA (final concentration) and 10 mg/ml of DNase (final concentration). Cells are washed once with 5% BSA in APBS, then incubated in 1% BSA in APBS with 0.1 mM PMSF and 0.05 mM TLCK (both protein inhibitors) for 10 min on ice. After an additional wash, cells are put back in culture for 4 h at 26 °C in either ASF medium alone or in medium with 1 μg/ml of brefeldin A (BFA).

4.3. Some concluding remarks

With the discovery of Toll-like receptors in mammals [62,63], it has become clear that a complex set of interactions and integration exists between the ancestral innate immune system and the phylogenetically more recent adaptive immune system of vertebrates. Increasing evidence suggests that hsps may play an important role at the interface of these two systems [64]. The availability and relative ease of maintaining MHC-defined strains

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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