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A natural heterohaemagglutinin in *Xenopus laevis* serum

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Summary. In most adult *Xenopus laevis* the serum contains a 'natural' factor capable of lysing the erythrocytes from a wide variety of amniote species. The factor has no effect on the erythrocytes of another amphibian, *Ambystoma mexicanum*, nor will serum from one animal lyse red cells from another *Xenopus* individual. No lysing factor was present in the serum of larval (tadpole) *Xenopus*.

Heating of *Xenopus* serum to 56° for 30 min, absorption of the serum with zymosan or inulin, or removal of calcium and magnesium ions results in loss of lytic activity, although haemagglutinating activity remains, suggesting that the factor can fix complement. The factor elutes from a gel chromatography column in the 19S peak, and is inactivated by thiol reduction and subsequent alkylation.

These findings, coupled with immunoabsorption studies suggest that the haemagglutinin is an immunoglobulin of the IgM class. The significance of this suggestion is discussed in the light of previous reports of 'natural' heterohaemagglutinins in other species.

INTRODUCTION

The presence in normal amphibian sera of agglutinins to the erythrocytes of foreign species has been known since Noguchi (1902) demonstrated lytic activity to red cells by the sera of the bullfrog, *Rana catesbeiana*, and the 'ditch-snake', *Amphiuma means*. More recently Frair (1963) showed that normal *Bufo marinus* serum from four individuals agglutinated one out of three snapping turtle (*Chelhydra serpentina*) erythrocyte samples. In 1976, Balding & Gold reported a natural heterohaemagglutinin for human red cells in the serum of the toad *Bufo regularis*; this serum had an anti-(B+HP) specificity and, interestingly, did not appear to be an immunoglobulin (Ig). The heterohaemagglutinin was found to elute in the α_1-globulin region on cellulose acetate electrophoresis. Such non-Ig heterohaemagglutinins have also been described in cyclostomes, fishes and reptiles (reviewed by Gold & Balding, 1975, 1976). These authors use the term 'receptor specific proteins' for such non-Ig 'antibodies'.

Attempts to raise an anti-rat erythrocyte antibody in the South African Clawed Toad, *Xenopus laevis* (Daudin), were frustrated at the outset by the presence of a lytic factor to rat erythrocytes in the control sera extracted from uninjected toads on day 0 of the immunization schedule. This paper reports an investigation of the factor in an attempt to characterize it more fully.

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MATERIALS AND METHODS

Animals

Mature adult *Xenopus laevis*, axolotls (*Ambystoma mexicanum*) and green lizards (*Lacerta viridis*) were purchased from T. Gerrard & Co., Littlehampton, West Sussex. Tokay geckoes (*Gekko gecko*) were bought from Menagerie, Weybridge, Surrey: adult chicken (*Gallus domesticus*) were obtained from

Greenacre Farm, Colchester. Mammals and *Xenopus* tadpoles came from our own breeding colonies.

Blood samples

Samples of blood were collected in 0·2 vols of 3·5% tri-sodium citrate. Adult amphibians were bled by cardiac puncture while under tricaine methane sulphonate ('MS 222'—Sandoz Products, Ltd., London) anaesthesia. *Lacerta*, *Gekko*, guinea-pig, rat, mouse and Syrian hamster blood samples were obtained by cardiac puncture while the animals were anaesthetized with 'Fluothane' (Halothane, B.P., Imperial Chemicals Industries, Ltd., Macclesfield). Rabbits were bled from the marginal ear vein; chickens were bled from a wing vein at the elbow joint. Samples of sheep blood in Alsever's solution and horse blood (oxalated) were purchased direct from Flow Laboratories, Irvine, Scotland and Oxoid Ltd., London respectively.

Blood samples were centrifuged for 10 min at 500 *g*, and were then resuspended in 10 times the original blood volume of NaCl solution (6·6 g/l for amphibian blood, 7·5 g/l for reptile blood, and 8·5 g/l for chicken and mammal blood) before further centrifugation for 10 min at 500 *g*. Resuspension in NaCl solution and centrifugation were thrice repeated before a final resuspension in NaCl solution such that the red cell concentration was 10^8 ml^{-1}.

Red cells were treated with papain (Sigma Chemical Co. Ltd., London) after the method of Dunsford & Bowley (1967).

Xenopus serum

Adult *Xenopus* serum was prepared as previously described (Jurd & Stevenson, 1974); tadpole serum was obtained as described in Jurd *et al.* (1975). Serum samples from different adults were kept separate and, where necessary, were stored at $-10°$. Tadpole serum was pooled. If so required, *Xenopus* serum was decomplemented by heating for 30 min at 56°.

Testing of Xenopus sera for lytic or agglutinating activity

0·05 ml samples of red cell suspension were pipetted into the round-bottomed wells of polyvinyl chloride microtitration plates ('Microtiter Plates'—Cooke Laboratory Products, Alexandria, Virginia, U.S.A.); 0·05 ml of doubling dilutions (in the relevant isotonic saline solution for the respective red cells) of non-heat inactivated serum was added. Serum and cell suspension were mixed before standing for 2 h when the presence or absence of red cell lysis was noted. 0·05 ml samples of doubling dilutions of heat inactivated *Xenopus* serum were mixed with further 0·05 ml samples of red cell suspension. The maximum dilutions at which lysis or agglutination was observable after 2 h were recorded. Tests were carried out at various temperatures between 0° and 37°.

Treatment of serum with thiols and alkylation

Heat-inactivated *Xenopus* serum was diluted times two with 0·02 M sodium phosphate + 0·11 M NaCl buffered at pH 7·3 (phosphate-buffered isotonic saline—PBS). Four 2 ml samples were respectively dialyzed at 4° against 1000 vols of (i) PBS for 24 h; (ii) 0·1 M 2-mercaptoethanol in PBS (or 0·01 M dithiothreitol) for 2 h and then against 3 changes of PBS; (iii) 0·1 M 2-mercaptoethanol (or 0·01 M dithiothreitol) in PBS for 2 h, then 3 changes of PBS for 24 h each, then 0·05 M iodoacetamide in PBS for 4 h, followed by 2 changes of PBS for 24 h each, and (iv) 0·05 M iodoacetamide for 4 h, followed by 2 changes of PBS for 24 h each.

Gel chromatography

3 ml samples of *Xenopus* serum were passed through a downward flowing Sephadex G150 (Pharmacia Ltd., Uppsala, Sweden) column, 9 × 600 mm, eluting with a 0·2 M Tris-HCl buffer. Eluant fractions were assayed for protein content by optical absorption at 280 nm. Column samples were concentrated to a protein concentration of 1 mg/ml in a 'Minicon' B15 concentrator (Amicon Corporation, Lexington, Mass., U.S.A.).

Immunoelectrophoresis

Immunoelectrophoresis was carried out using a Millipore Immunoagaroslide (Millipore U.K., Ltd., London) system. Protein samples were electrophoresed for 25 min in 0·07 M sodium barbital + 0·05 M boric acid + 0·01 M sodium phosphate buffered at pH 8·6. After reaction with antiserum for 24 h, gels were deproteinized and stained with amido-black according to the manufacturers' instructions.

Preparation of antisera

Recent studies (reviewed by Atwell & Marchalonis, 1976) on anuran Igs suggest that the anuran 7S Ig has a heavy chain not homologous in amino-acid

sequence or electrophoretic mobility to the γ chain of mammalian IgG: thus the use of the term 'IgG' for the 7S Ig of *Xenopus* (as in Jurd & Stevenson, 1974) should be discontinued. The term suggested by Atwell & Marchalonis (1976), 'IgRAA' (for 'Immunoglobulin-Reptilia-Amphibia-Aves'), used to describe the non-μ chain-containing 7S Ig, is adopted here in place of the 'IgG' previously used.)

Immunoelectrophoretically pure IgM, IgRAA, and *Xenopus* light chain were prepared from *Xenopus* serum as described by Jurd & Stevenson (1974). Antisera to *Xenopus* whole serum, IgM, IgRAA and *Xenopus* light chain respectively were raised in New Zealand rabbits, also as described in Jurd & Stevenson (1974). The IgG fractions were prepared from the rabbit antisera using the method of Stevenson & Dorrington (1970).

IgG from the rabbit anti-*Xenopus* IgM serum was absorbed with *Xenopus* IgRAA, as outlined previously (Jurd & Stevenson, 1976) to yield an anti-μ chain preparation. IgG from the rabbit anti-*Xenopus* IgRAA was similarly absorbed with *Xenopus* IgM to give anti-(IgRAA heavy chain). The purity of these preparations was tested by immunoelectrophoresis.

Absorption of Xenopus whole serum

Samples of *Xenopus* serum were absorbed for 24 h at 4° with doubling dilutions of IgG from rabbit anti-*Xenopus* IgM, anti-IgRAA, anti-light chain,

Table 1. Lytic and agglutinating activity of *Xenopus* serum against red blood cells of different species. All tests carried out at room temperature

	(a) Log_2 (max. dilution non-heat inactivated serum giving lysis) Enzyme						(b) Log_2 (max. dilution heat-inactivated serum giving agglutination) Enzyme					
	Treated			Untreated			Treated			Untreated		
	Toad						Toad					
Red cell species	1	2	3	1	2	3	1	2	3	1	2	3
Ambystoma mexicanum (axolotl)	No lysis						No agglutination					
Lacerta viridis (green lizard)	7	7	8	4	3	4	10	8	9	6	4	5
Gekko gecko (Tokay gecko)	N.D.			3	5	4	N.D.			5	5	4
Gallus domesticus (chicken)	8	8	9	5	4	5	8	8	7	5	5	6
Oryctolagus cuniculus (New Zealand rabbit)	7	8	7	4	4	4	5	8	7	4	5	4
Oryctolagus cuniculus (Dutch rabbit)	7	8	6	5	4	3	8	9	7	5	5	3
Rattus norvegicus (Wistar rat)	8	8	6	4	4	3	8	8	8	4	5	4
Mus musculus (BALB/c mouse)	9	8	7	5	5	6	9	10	8	5	6	6
Mus musculus (NZB mouse)	8	8	7	4	5	5	9	10	8	5	5	5
Cavia porcellus (guinea-pig)	9	8	9	6	4	6	10	8	9	7	5	6
Mesocricetus auratus (Syrian hamster)	7	8	6	4	5	5	8	8	9	4	5	4
Equus caballus (horse)	6	8	7	3	5	4	7	8	9	3	5	6
Ovis argal (sheep)	7	8	9	5	6	6	8	9	9	5	6	6

N.D. = not done.

anti-μ chain and anti-(IgRAA heavy chain) respectively. After absorption the serum was centrifuged for 60 min at 20,000 *g* and the supernatants were tested against further samples of the relevant absorbing antibodies by agar-gel immunodiffusion (Ouchterlony, 1949) for the presence of residual antigen. The minimum amount of antibody needed to absorb totally the relevant antigen was thus ascertained.

RESULTS

Lytic and agglutinating activity of *Xenopus* serum to xenogeneic red cells

The effect of adding 0·05 ml of various dilutions of non-heat inactivated *Xenopus* serum from 3 toads to 0·05 ml samples of papain-treated or papain-untreated erythrocyte suspensions (containing 5×10^6 cells) from foreign species is shown in column (a) of Table 1. It was found that *Xenopus* serum would lyse the red cells of all species tested except those of the urodele amphibian *Ambystoma mexicanum*. The lytic titre is noticeably higher with enzyme-treated red cells.

Column (b) indicates the maximum dilution of heat-inactivated *Xenopus* serum (from the same 3 animals) which would agglutinate red cells within 2 h. Again it was found that the *Xenopus* serum would agglutinate the erythrocytes of all the amniote species tested, but not those of *Ambystoma*; again the agglutinating titre was higher when papain-treated cells were used as targets.

Table 2 shows the effect of temperature on the lytic and agglutinating activity of serum from 2 *Xenopus* on Wistar rat red cells. It was noticeable that both agglutination and lysis occurred at 0°.

Addition of 0·02 ml of fresh BALB/c mouse serum, fresh rabbit serum or fresh guinea-pig serum to the erythrocyte/heat-inactivated *Xenopus* serum mixture did not result in lysis of the red cells, suggesting that although complement may be implicated in the lytic process initiated by non-heat inactivated serum, *Xenopus* agglutinating factor could not 'fix' mammalian complement to effect cell lysis.

Treatment of non-heat inactivated *Xenopus* serum with inulin or zymosan (which, in mammals, binds to the C3 component of complement) resulted in a loss of lytic activity, but not of agglutinating activity. Treatment of *Xenopus* serum with ethylene-diamine-tetra-acetic acid (EDTA) also resulted in a loss of lytic activity: this could be restored by the addition of calcium and magnesium to the serum when the EDTA had been dialysed out.

Dialysis of samples of non-heat inactivated *Xenopus* serum against an NaCl solution, or against an NaCl solution enriched with magnesium, or calcium, or magnesium and calcium resulted in a loss of lytic activity but not agglutinating activity towards rat cells in all serum samples except those dialysed against saline with both calcium and magnesium present (where lytic activity remained).

Table 2. Lytic and agglutinating activity of *Xenopus* serum against rat red cells at different temperatures

Temperature (°C)	Log_2 (max. dilution non-heat inactivated serum giving lysis) Enzyme Treated Toad 1	Treated Toad 2	Untreated Toad 1	Untreated Toad 2	Log_2 (max. dilution heat-inactivated serum giving agglutination) Enzyme Treated Toad 1	Treated Toad 2	Untreated Toad 1	Untreated Toad 2
0	6	7	3	4	7	9	4	5
4	6	7	4	4	8	8	5	5
10	7	7	4	4	8	8	4	6
20	7	6	4	4	8	9	5	6
28	7	7	5	5	7	9	5	6
33	7	6	3	5	8	8	5	5
37	8	7	4	4	8	8	5	6

These results strengthen the view that complement, dependent on the presence of Mg^{++} and Ca^{++}, and exhibiting at least some of the features of mammalian complement, is present in *Xenopus* serum and is implicated in the lytic activity of the natural agglutinin.

0·5 ml samples of heat-inactivated *Xenopus* serum were absorbed with 0·5 ml of packed Wistar rat red cells in 3 ml tubes in a shaking water-bath for 2 h at 20°. After centrifuging down the agglutinated red cells the supernatant serum was tested against rat, rabbit, mouse and chicken red cells. In no instance was agglutination observed. It did not prove possible to elute the 'antibody' from the absorbing, agglutinated rat cells by treatment with either 0·85% NaCl solution or 0·1 M NH_4OH in 0·85% NaCl (testing the latter after dialysis against NaCl to remove the ammonia).

To test the universality of the lysing/agglutinating factor in *Xenopus* serum, serum samples from 23 toads were tested against Wistar rat red cells. Sera from 19 of the toads lysed the rat red cells. The 4 'ineffectual' serum samples were found to be equally ineffective against rabbit and sheep red cells.

Three samples of pooled tadpole serum, each from 10 animals, were found to possess no lytic or, after heat inactivation, agglutinating activity to rat, rabbit, sheep or chicken erythrocytes.

Lytic and agglutinating activity of *Xenopus* serum to allogeneic red cells

Heat-inactivated serum samples from each of 10 adult *Xenopus* were tested against the red cells from the 9 other toads. No agglutinating activity to such allogeneic red cells was demonstrable.

Effect of reduction and subsequent alkylation on the agglutinating factor

Treatment of heat-inactivated adult *Xenopus* serum with either 2-mercaptoethanol or dithiothreitol, followed by alkylation with iodoacetamide resulted in a total loss of agglutinating activity towards rat and rabbit erythrocytes.

Control experiments in which serum was simply dialysed against PBS, was treated with thiol but was not subsequently alkylated, or was treated with iodoacetamide alone did not impare agglutinating activity.

Identification of agglutinating factor

Gel chromatography. The elution profile obtained when adult *Xenopus* serum was passed down a Sephadex G150 column is shown by the closed circles in Fig. 1. The 3 peaks correspond to the 19S, 7S and 4S peaks recorded by Jurd *et al.* (1975). Column samples were concentrated to 1 mg protein

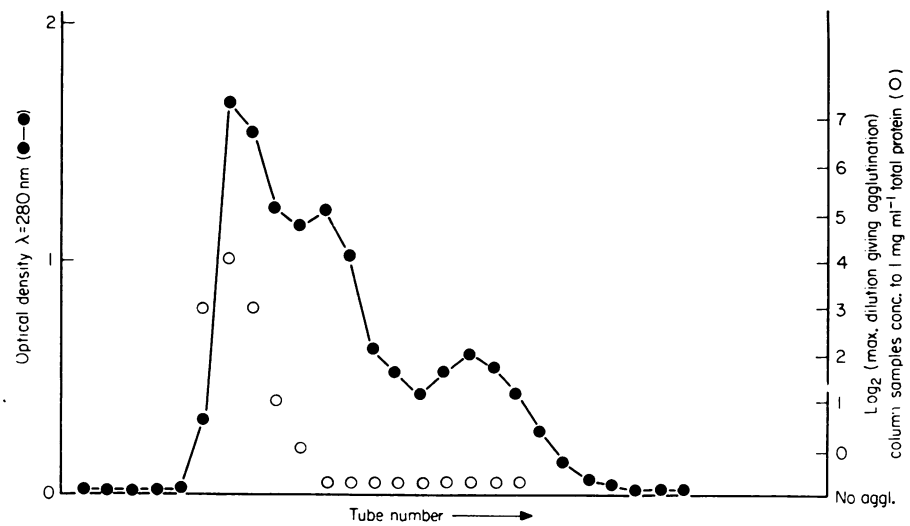

Figure 1. Elution profile for *Xenopus* serum passed down a Sephadex G150 column (closed circles): the 3 peaks correspond to the 19S, 7S and 4S peaks respectively, described by Jurd *et al.*, 1975. The open circles show agglutinating titres for eluate fractions concentrated to 1 mg ml^{-1} total protein concentration.

Table 3. Agglutinating activity of *Xenopus* serum absorbed with various antibody preparations

Antibody used to absorb *Xenopus* serum prior to test	Agglutinating activity towards	
	Rat red cells	Rabbit red cells
Anti-IgM	–	–
Anti-IgRAA	–	–
Anti-light chain	–	–
Anti-μ chain	–	–
Anti-(IgRAA heavy chain)	+	+
Anti-(IgRAA heavy chain) + anti-μ chain	–	–
(No absorption)	+	+

ml^{-1} and tested for agglutinating activity against an equal volume of Wistar rat red cells, concentration 10^8 cells ml^{-1}. Log_2 (maximum dilution of sample giving positive agglutination after 2 h) is shown by the open circles in Fig. 1. Note that the agglutinating activity is restricted to eluate in the first (19S) peak.

Immunoelectrophoresis of the fraction corresponding to the summit of the first peak yielded arcs in the α_1- and γ-globulin regions of the gel (as compared with reference sera).

Absorption with anti-Ig preparations

In an attempt to characterize further the agglutinating factor present in the 19S chromatography peak, samples of adult *Xenopus* serum (heat-inactivated) and 19S peak eluate were each absorbed with anti-IgM, anti-IgRAA, anti-*Xenopus* light chain, anti-μ chain, and anti-(IgRAA heavy chain) to remove the relevant antigen. The absorbed sera (or eluates) were tested against rabbit or rat red cells. The presence or absence of agglutination for absorbed serum is shown in Table 3. Note that the serum absorbed with anti-light chain, anti-IgRAA, anti-IgM and anti-μ chain will not agglutinate erythrocytes, but agglutinating activity remains in serum absorbed with anti-(IgRAA heavy chain). Absorption of eluate gave identical results.

DISCUSSION

Nineteen out of 23 adult *Xenopus laevis* tested have present in their serum a 'natural' factor or factors capable of lysing rat red blood cells. Further tests were carried out with serum samples from 3 of the 19 toads. The sera were found to lyse red cells from the green lizard, from the Tokay gecko, from the domestic chicken and from each of a variety of mammalian species. The sera would not lyse axolotl (*A. mexicanum*) cells. Heat inactivation of the sera at 56° for 30 min resulted in loss of lytic activity, as did treatment with inulin, zymosan, or removal of Ca^{++} and Mg^{++} ions, although in each case agglutinating activity remained. By parallels with the mammalian immune response this suggests that the agglutinating factor (or heterohaemagglutinin) can 'fix' complement, resulting in lysis of the red cells: complement is heat-labile at 56°. Lytic activity could not be restored by the addition of fresh mouse, guinea-pig or rabbit serum, implying the possible existence of specific, amphibian complement components or pathways. Similar implications have been found by Chiller *et al.* (1969) working with fish complement: this group discovered that for its effective action, the complement must be derived from a fish species closely related to the species from which agglutinating antibody was taken. The lytic and agglutinating activity of non-heat inactivated and heat-inactivated sera respectively, was effective at all temperatures tested between 0° and 37°: the effects at low temperatures are perhaps a reflection of the 'cold adapted' immune response of a poikilothermic animal.

It was not possible to demonstrate either lytic or agglutinating activity to xenogeneic red cells in *Xenopus* tadpole serum. Isoagglutinating factors could not be found either when serum samples from each of 10 adult toads were tested against red cells from the other 9.

The heterohaemagglutinin from the adult toads eluted from a chromatography column in the 19S fraction, and was found to be sensitive to reduction by thiols. These observations, together with its apparent ability to fix complement, suggested that the heterohaemagglutinin might be an IgM on the basis that *Xenopus* IgM has an $S_{20,w}^0 = 19{\cdot}5S$ (Jurd & Stevenson, 1974) and is thiol-sensitive (Hadji-Azimi, 1971). Absorption of the *Xenopus* serum with relevant antibodies seems to confirm that the heterohaemagglutinin is an IgM. Absorption with anti-IgM, anti-light chain, anti-μ chain and anti-IgRAA (which will react with the light chains of IgM) results in the loss of agglutinating activity,

whereas absorption with anti-(IgRAA heavy chain) has no effect.

The presence in adult *Xenopus* serum of a 'natural' heterohaemagglutinin to xenogeneic red blood cells is of interest. Unlike many of the lower vertebrate heterohaemagglutinins hitherto described (Gold & Balding, 1975, 1976; Balding & Gold, 1976), the *Xenopus* haemagglutinin is an immunoglobulin of the IgM class with its polypeptide chains linked by disulphide bonds. It thus seems reasonable to call it a 'natural antibody'. It is interesting that the immunoabsorption studies showed no other haemagglutinin, such as the α_1-macroglobulin described in *Bufo regularis* (Balding & Gold, 1976), the heavy (mol. wt 280,000), non-Ig binding protein of the nurse shark *Ginglymostoma cirratum* (Harisdangkul *et al.*, 1972a, b) or the thiol-resistant heterohaemagglutinin of *Alligator mississippiensis* (Gold, 1976), to be present in *Xenopus* serum. However, IgM 'natural antibodies' are also produced in *G. cirratum* (Rudokoff & Sigel, 1970; Rudikoff *et al.*, 1970), together with 7S Ig haemagglutinin (Clem *et al.*, 1967). IgM natural antibodies may also, on indirect evidence, be present in the mouse (Potter, 1971).

It is thus possible that both Ig natural antibodies and non-Ig receptor specific proteins may co-exist in some species but that in other species or groups either the former or the latter has disappeared. In *Xenopus* no non-Ig heterohaemagglutinin was found, suggesting that in this animal possible non-Ig haemagglutinins in ancestral forms may have been lost in the course of evolution: anurans are thought to lie on a branch away from the main trunk of vertebrate phylogenesis (Porter, 1972), and the Pipidae (the family to which *Xenopus* belongs) itself represents a fairly specialized group within the Anura (Noble, 1931). The possibility of non-Ig receptor specific proteins in *Xenopus* which are not haemagglutinins must be borne in mind.

The reason for the existence of the natural antibodies in *Xenopus* is problematical. Their lytic activity (in the presence of complement) towards amniote red cells, but not axolotl red cells suggests that the amniote cells share a common receptor, or antigen, absent on the urodele cells. It should be noted that absorption of *Xenopus* serum with rat red cells resulted in a loss of agglutinating activity towards chicken, rabbit and mouse red cells, reinforcing the view that such a common antigen exists on amniote erythrocytes. That 4 of the 23 *Xenopus* sera tested did not exhibit lytic activity may indicate that the amniote red cell antigen resembles a naturally-occurring antigen usually, but not always encountered during larval or early adult life: no heterohaemagglutinin was found in tadpole serum. One might speculate that the antigen is first met in the gut when the animal changes to a carnivorous diet at metamorphosis. Many of the non-Ig heterohaemagglutinins have sugar specificities (Bezkorovainy *et al.*, 1971; Harisdangkul *et al.*, 1972a, b; Balding & Gold, 1976): the nature of the antigen to the *Xenopus* IgM heterohaemagglutinin is currently under investigation.

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