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Detection and characterization of natural and inducible lectins in human serum

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A B S T R A C T

This study was performed to detect and characterise the possible occurrence of natural and inducible lectins in human serum by hemagglutination method, wherein, the serum was treated using exogenous elicitors, namely, proteases and detergents.

Natural and inducible lectins were detected and characterised in human serum. Untreated serum agglutinated buffalo and rabbit RBC, while serum treated with pronase, trypsin, α-chymotrypsin or SDS *for the very first time,* agglutinated hen/hen and sheep RBC within 15 min in a dosimetric manner. Cross adsorption test revealed that both trypsin and α-chymotrypsin-treated serum showed similar RBC adsorption pattern. The lectin activity in untreated, pronase-treated serum was cation independent and moderately sensitive/insensitive to calcium chelator EDTA, whereas, trypsin-treated serum was cation dependent as well as EDTA sensitive (sheep RBC), cation independent and EDTA insensitive (hen RBC). Hemagglutination of untreated serum was inhibited by certain glycosides and di-, oligo-saccharides, whereas, activity in pronase-treated serum was inhibited by hexosamines. By contrast, hemagglutination of trypsin-treated serum showed specificity for acetylated mannosamine as well as sialic acid for sheep RBC and certain glycoproteins for hen RBC.

Thus, we have detected inducible lectins with distinct ligand binding specificity, upon treatment of human serum with proteases, namely, pronase and trypsin. Nevertheless, lectin activity was found in untreated human serum too with different ligand specificity.

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1. Introduction

Lectins are conventionally defined as proteins/glycoproteins of non-immune origin with an unique ability to specifically and reversibly bind to carbohydrate structures present on cell surfaces, extracellular matrices, or secreted glycoproteins [1,2]. They are ubiquitously distributed in microbes [3], plants [4] as well as animals and humans [5–7]. In humans, about 49 distinct types of lectins including selectins and galectins were reported to occur in various cells, tissues, organs and in blood plasma/serum [8–14]. Six naturally occurring lectins have been detected in human blood, that includes, C-reactive protein (CRP), serum amyloid protein (SAP), H-ficolin, mannan-binding lectin (MBL), tetranectin and L-ficolin. However, none of these humoral lectins were detectable in crude serum by hemagglutination.

Isolated CRP, SAP and H-ficolin could agglutinate, respectively, pneumococcal capsular polysaccharide-coated sheep RBC [15], complement-coated sheep RBC [16] and bacterial lipopolysaccharide-coated human RBC [17]. Only Hamazaki [18] reported that isolated SAP can agglutinate horse and rat RBC. These humoral lectins, with an exception of H-ficolin [17] required Ca^{2+} to bind various appropriate ligands. Indeed, few conflicting reports indicate the divalent cation independent activity of CRP [19,20], tetranectin [21] and L-ficolin [22,23]. These lectins bind to diverse simple to complex ligands, but predominantly, *N*-acetylgalactosamine, *N*-acetylglucosamine, phosphoryl choline, heparin, mannan and plasminogen can be considered to be the best ligands for H-ficolin, L-ficolin, CRP, SAP, MBL and tetranectin, respectively [17,22,24–28]. All these lectins could activate complement system as well as mediate opsonophagocytosis by macrophages and/or neutrophils. H-ficolin could interact directly with pathogenic bacteria and effectively abrogate their growth. Apart from this lectin-mediated immune responses, the treatment of various biochemical constituents with endogenous or exogenous agents, result in generation of new immunologically relevant molecules which could possibly augment the existing capacity of host immune responsiveness.

Generation of potent antimicrobial activity from lactoferrin, casein, albumin, egg white lysozyme and ovalbumin [29–33] has been reported upon treatment with exogenous proteases. Furthermore, lectin activity could be generated from egg white lysozyme after chemical treatment [34]. Immunological functions

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of inducible lectins have been demonstrated in various animal models. Investigations as reported above are not observed in human serum till date and thus we have explored the possibility for generation of immunologically reactive molecules. Furthermore, it is also that these lectins generated by proteases (specifically microbial protease) may have immunological functions, because there are loads of microbes in our body that can produce proteases into our system.

Representatives of proteases from different classes (serine, aspartic, cysteine and non-specific) and detergents (anionic and cationic), were chosen randomly for our initial analysis. Besides, it is also clear from previous reports that some of these proteases as well as SDS are known to elicit certain activity in various lower animals and lectins obtained from induction of human serum have not been reported till date. Hence, in the present study, we detected and characterised generation of lectins in human serum for the *very first time* upon treatment with exogenous proteases and detergents. Pure natural lectins were not tested, but, the hemagglutinating activity and characteristics of untreated serum was also extensively investigated for the *first time* in order to compare with the treated serum.

2. Materials and methods

2.1. Materials

Trypsin, pronase, pepsin, phenylmethylsulphonyl fluoride (PMSF), amino acids as well as their derivatives, glycoproteins such as bovine sub-maxillary mucin, asialo-BSM, fetuin, asialo-fetuin, thyroglobulin, mucin, ovalbumin and phosphoryl choline were purchased from Sigma chemical (Co., St Louis, USA). Papain and α-chymotrypsin were obtained from SRL and Himedia, Mumbai, India, respectively. Carbohydrates (mono-, di-, oligo- and polysaccharides) were procured from BDH, Fluka, Serva, Merck, Himedia and Sigma. All other chemicals and reagents used in this study were of the highest analytical grade purchased from local agencies.

2.2. Tris-buffered saline (TBS)

Eight different types of tris-buffered saline (TBS) containing 0.02% sodium azide were prepared as listed below and stored at 10 °C. The osmolalities were determined using Cryoscopic Osmometer (Osmomat 030, Gonotec, Germany).

TBS-I (50 mM Tris, 50 mM NaCl, 50 mM $CaCl_2$, pH 7.5, 300 mOsm), TBS-II (50 mM Tris, 110 mM NaCl, pH 7.5, 300 mOsm), TBS-III (50 mM Tris, 115 mM NaCl, 10 mM $CaCl_2$, pH 7.5, 300 mOsm), TBS-IV (50 mM Tris, 97 mM NaCl, 25 mM EDTA, pH 7.5, 300 mOsm), TBS-V (50 mM Tris, 25 mM NaCl, 10 mM $CaCl_2$, pH 7.5, 135 mOsm), TBS-VI (50 mM Tris, 90 mM NaCl, 10 mM $MgCl_2$, pH 7.5, 300 mOsm), TBS-VII (50 mM Tris, 95 mM NaCl, 10 mM $SrCl_2$, pH 7.5, 300 mOsm) and TBS-VIII (50 mM Tris, 110 mM NaCl, 1 mM $MnCl_2$, pH 7.5, 300 mOsm).

2.3. Phenylmethylsulphonyl fluoride (250 mM)

43.5 mg phenylmethylsulphonyl fluoride was dissolved in 1 ml isopropanol and various concentrations were obtained by appropriate dilution with TBS-I.

2.4. Collection of samples

Human blood and serum samples were obtained from Voluntary Health Service, Taramani, Chennai and Lions Blood Bank, Egmore, Chennai. Serum samples were diluted with an equal volume of 0.9% physiological saline containing 0.02% sodium azide and stored at −25 °C, until use. Human serum of blood group AB was used in all these assays, unless otherwise mentioned.

2.5. Vertebrate blood samples

Blood samples were obtained from various vertebrates listed below:

Sl. no.	Common name	Scientific name	Source
1.	Human	*Homo sapiens*	Blood banks
2.	Rabbit	*Oryctolagus cuniculus*	*Our laboratory*
3.	Rat	*Rattus norvegicus*	
4.	Mouse	*Mus musculus*	
5.	Buffalo	*Bubalus bubalis*	Chennai corporation slaughter house Perambur, Chennai
6.	Ox	*Bos indicus*	
7.	Sheep	*Ovis aries*	
8.	Goat	*Capra aegagrus hircus*	
9.	Hen	*Gallus gallus domesticus*	*Local chicken stalls, Chennai*

All blood sample collections performed in the laboratory were approved by Institutional Animal Ethical Committee (IAEC), India, guidelines (360/01/a/CPCSEA). Samples were collected in Alsever's solution containing 100 μg/ml streptomycin and stored at 10 °C.

2.6. Erythrocytes (RBC)

RBC were prepared by following the method of Maheswari et al. [35], by centrifugation (400 × *g* for 5 min at 28 °C) and pellet was finally suspended in respective buffers as 1.5% (v/v) RBC suspension. Hen and sheep RBC prepared as mentioned above were packed into 500 μl pellets and used for cross adsorption test.

Note: Centrifugation was performed as mentioned above throughout this study.

2.7. Fixation of RBC

Prepared RBC was fixed by suspending the RBC pellet in PBS (0.1 M, pH 7.2) containing 5% formaldehyde for 24 h at 10 °C. The fixed RBC were extensively washed in saline and resuspended in TBS-III before use.

2.8. Hemagglutination assays

The hemagglutination (HA) assays were routinely performed in V-bottom microtiter plates (Greiner, Nürtingen, Germany) by serial two-fold dilution of 25 μl test samples (untreated or treated-sera samples) against various vertebrate RBC types following the method of Maheswari et al. [35] and that of Garvey et al. [36].

2.9. Induction of hemagglutinating activity in serum by various proteases/detergents

500 μl sera (blood group AB) were mixed with equal volumes of each one of the freshly prepared five proteases including pronase,

trypsin, α-chymotrypsin, pepsin and papain (prepared 1 mg/ml in TBS-I) or one of the three detergent solutions, namely, SDS, Tween 20 and Triton X-100 (4 mg/ml in TBS-III), separately and incubated at 37 °C for 90 min. In controls, serum samples were concurrently incubated with equal volumes of TBS-I (for proteases) and TBS-III (for detergents). All these test samples were centrifuged and the supernatants were tested for HA activity against various vertebrate RBC types. Detergents-treated sera samples were analysed for HA using fixed RBC types. Induction of pronase/SDS-induced hemagglutinating activity was also analysed in sera of blood groups A, B, and O.

2.10. Determination of role of pronase

100 μl serum was treated with an equal volume of heat-inactivated pronase (1 mg/ml; heated for 15 min at 100 °C in a water bath, centrifuged and supernatant used) and tested for HA activity against hen RBC.

2.11. Pronase inhibition assay

100 μl pronase (2 mg/ml) was pre-incubated with an equal volume of PMSF or EDTA at different concentrations (prepared in TBS-I) for 60 min at 28 °C. To this mixture, 200 μl serum was added and HA activity was tested against hen RBC.

2.12. Cross adsorption test

This assay was performed using 500 μl each of trypsin- and α-chymotrypsin-treated serum samples with equal volumes of both sheep as well as hen RBC pellets separately, following the method of Maheswari et al. [35] and the residual activity was analysed against both the RBC types.

2.13. Time course analysis

500 μl sera were treated with equal volumes of pronase, trypsin or SDS, separately. 100 μl was collected at various time points, centrifuged and the supernatants were assayed for HA activity. Pronase- or SDS-treated samples were tested using only hen RBC, whereas, trypsin-treated samples were analysed against both hen and sheep RBC.

2.14. Dosimetric analysis

100 μl sera were mixed with an equal volume of different concentrations of pronase or SDS solutions and incubated for 30 min at 37 °C. Each reaction mixture was centrifuged and the supernatant was assayed for HA activity against hen RBC.

2.15. Divalent cation dependency and EDTA sensitivity

The naturally occurring HA activity in serum was determined against buffalo and rabbit RBC by two-fold serial dilution of 25 μl serum sample with different buffers, namely, TBS-I, TBS-II and TBS-IV. The HA activity of trypsin-treated serum was analysed against both hen and sheep RBC using these same buffers. The HA activity of pronase-treated serum was analysed against hen RBC extensively in TBS-II, III, IV, VI, VII and VIII. (RBC suspensions were prepared in the respective buffers).

2.16. Hemagglutination-inhibition assays

Hemagglutination-inhibition assay was performed by following the method of Maheswari et al. [35]. Several carbohydrates (prepared in TBS-V), a phospholipid (prepared in TBS-I), aminoacids and their derivatives (prepared in TBS-V) and glycoproteins (prepared in TBS-III) were tested for their ability to inhibit the natural, pronase- and trypsin-inducible HA activity in serum against respective RBC types. pH of these test solutions were adjusted to pH 7.5 using NaOH pellets.

3. Results

3.1. Hemagglutination profile of untreated serum

All serum samples (blood groups A, B, O and AB) agglutinated buffalo and rabbit RBC with a titer of 16, rat and mouse RBC with the titer of 8, whereas, they never agglutinated ox, sheep, goat and hen RBC (Table 1).

3.2. Hemagglutination profile of serum treated with proteases/detergents

Among five proteases tested only pronase, trypsin and α-chymotrypsin generated hemagglutinating activity in serum (Table 2). Serum samples treated with pronase at a concentration of 100 or 500 μg/ml agglutinated hen RBC with a titer of 256, whereas, the serum treated with trypsin or α-chymotrypsin at a concentration of only 500 μg/ml were found to agglutinate both sheep and hen RBC with the titers of 64 and 128, respectively.

Among the three detergents, only SDS could generate HA activity in the serum against all the four human RBC types as well as ox, sheep and hen RBC, with the highest titer of 128 for the avian RBC (Table 3).

Hemagglutination profile of pronase and SDS-treated serum (blood groups A, B and O) gave similar hemagglutination profiles and agglutinated hen RBC with highest titer of 256 (Tables 4 and 5).

Similar HA profiles were observed with sera samples obtained naturally and after recalcification of citrated whole blood or plasma (data not shown). Furthermore, pre-treatment of hen RBC with pronase or SDS; hen and sheep RBC with trypsin did not make them susceptible to agglutination by untreated sera (data not shown). Besides, pronase, SDS or trypsin by itself did not agglutinate hen or sheep RBC (data not shown). Pronase/trypsin-treated samples were stored at 10 °C, wherein, the HA activity generated in the sera treated with pronase remained stable even after 3 weeks, interestingly, the trypsin-treated sera samples after 24 h retained the same high titer of

Table 1
Hemagglutinating activity of untreated serum against various vertebrate RBC types.

Blood group identity of serum	Serum hemagglutination titer against various RBC types[a]											
	Human A	Human B	Human O	Human AB	Buffalo	Rabbit	Rat	Mouse	Ox	Sheep	Goat	Hen
A	0	8	0	0	16	16	8	8	0	0	0	0
B	8	0	0	0	16	16	8	8	0	0	0	0
O	16	8	0	8	16	16	8	8	0	0	0	0
AB	0	0	0	0	16	16	8	8	0	0	0	0

[a] Data represent median titer values from six determinations for each RBC type using different stocks of pooled serum (single and multiple individuals).

Table 2
Hemagglutinating activity of serum (AB) treated with proteases against various vertebrate RBC types.

Serum treatment	Concentration of proteases (μg/ml)[a]	Hemagglutination titer against various RBC types[b]											
		Human A	Human B	Human O	Human AB	Buffalo	Rabbit	Rat	Mouse	Ox	Sheep	Goat	Hen
None		0	0	0	0	16	16	8	8	0	0	0	0
Pronase	100	0	0	0	0	4	8	8	8	0	0	0	256
	500	0	0	0	0	4	8	8	8	0	0	0	256
Trypsin	100	0	0	0	0	8	8	8	4	0	0	0	0
	500	0	0	0	0	8	8	8	4	0	64	0	128
α-Chymotrypsin	100	0	0	0	0	8	16	8	4	0	0	0	0
	500	0	0	0	0	8	16	8	4	0	64	0	128
Papain	100	0	0	0	0	8	16	8	4	0	0	0	0
	500	0	0	0	0	8	16	8	4	0	0	0	0
Pepsin	100	0	0	0	0	8	16	8	4	0	0	0	0
	500	0	0	0	0	8	16	8	4	0	0	0	0

[a] Final concentration in the reaction mixture.
[b] Data represent median titer values from eight determinations for each RBC type using different stocks of pooled serum (single and multiple individuals).

Table 3
Hemagglutinating activity of serum (AB) treated with detergents against various vertebrate RBC types.

Serum treatment	Hemagglutination titer against various RBC types[a]											
	Human A	Human B	Human O	Human AB	Buffalo	Rabbit	Rat	Mouse	Ox	Sheep	Goat	Hen
None	0	0	0	0	16	16	8	8	0	0	0	0
SDS	16	8	8	4	8	8	8	8	4	64	0	128
Triton X-100	0	0	0	0	8	8	8	8	0	0	0	0
Tween 20	0	0	0	0	8	8	8	8	0	0	0	0

[a] Data represent median titer values from three determinations for each RBC type using different stocks of pooled serum (single and multiple individuals).

Table 4
Hemagglutinating activity of serum treated with pronase against various vertebrate RBC types.

Serum samples tested	Hemagglutination titer against various RBC types[a]											
	Human A	Human B	Human O	Human AB	Buffalo	Rabbit	Rat	Mouse	Ox	Sheep	Goat	Hen
Serum A												
Untreated	0	8	0	0	16	16	8	4	0	0	0	0
Pronase-treated	0	8	0	0	2	16	64	2	0	0	0	128
Serum B												
Untreated	8	0	0	0	64	32	8	4	0	0	0	0
Pronase-treated	16	0	0	0	2	32	64	8	0	0	0	256
Serum O												
Untreated	8	8	0	4	16	32	16	4	0	0	0	0
Pronase-treated	16	16	0	8	64	64	16	4	0	0	0	256
Serum AB												
Untreated	0	0	0	0	16	16	8	8	0	0	0	0
Pronase-treated	0	0	0	0	4	8	8	8	0	0	0	256

[a] Data represent median titer values from eight determinations for each RBC type using different stocks of pooled serum (single and multiple individuals).

Table 5
Hemagglutinating activity of serum treated with SDS against various vertebrate RBC types.

Serum samples tested	Hemagglutination titer against various RBC types[a]											
	Human A	Human B	Human O	Human AB	Buffalo	Rabbit	Rat	Mouse	Ox	Sheep	Goat	Hen
Serum A												
Untreated	0	8	0	0	16	16	8	4	0	0	0	0
SDS-treated	0	0	4	8	4	8	8	4	8	16	0	256
Serum B												
Untreated	8	0	0	0	64	32	8	4	0	0	0	0
SDS-treated	0	0	2	16	4	8	8	4	8	16	0	256
Serum O												
Untreated	8	8	0	4	16	32	16	4	0	0	0	0
SDS-treated	16	16	16	16	4	4	8	4	4	32	0	256
Serum AB												
Untreated	0	0	0	0	16	8	8	8	0	0	0	0
SDS-treated	16	16	16	8	8	16	16	8	8	64	0	256

[a] Data represent median titer values from eight determinations for each RBC type using different stocks of pooled serum (single and multiple individuals).

256 for hen RBC, but the activity against sheep RBC declined to 0. SDS-induced serum HA activity was stable for three weeks when stored at 28 ± 2 °C (data not shown).

3.3. Role of pronase and inhibition of pronase action

Serum treated with heat-inactivated pronase failed to generate HA activity against hen RBC (Fig. 1), while that treated with active pronase was able to generate HA. As depicted in Figs. 2 and 3, dosimetric analysis of PMSF as well as EDTA revealed that at 12 and 50 mM respectively, generation of HA activity was not detectable in serum against hen RBC.

3.4. Cross adsorption test

The results presented in Table 6 shows that in both trypsin/α-chymotrypsin-treated serum samples, either of these two RBC types completely adsorbed its own hemagglutinating activity as well as the activity for the other RBC type after 3–4 sequential adsorptions.

3.5. Time course analysis

Pronase, trypsin or SDS-treated sera were found to agglutinate hen RBC within 15 min and was same even up to 120 min (Table 7). HA of trypsin-treated serum against sheep RBC too gave an identical profile (data not shown).

3.6. Dosimetric analysis

The generation of HA activity was detectable in pronase treated serum against hen RBC at 25 μg/ml (titer = 8), reached highest titer of 256 at 100 μg/ml (Fig. 4), whereas, the HA activity in SDS-treated serum was notable at 0.3 mg/ml (titer = 4) and reached the maximum titer at 2 mg/ml (Fig. 5).

3.7. Divalent cation dependency and EDTA sensitivity

The untreated serum gave a HA titer of 16 against buffalo and rabbit RBC in the presence of TBS-I or II. In the presence of TBS-IV the serum gave the titers of 8 and 4 against these two RBC types, respectively. Trypsin-treated serum gave a HA titer of 256 against

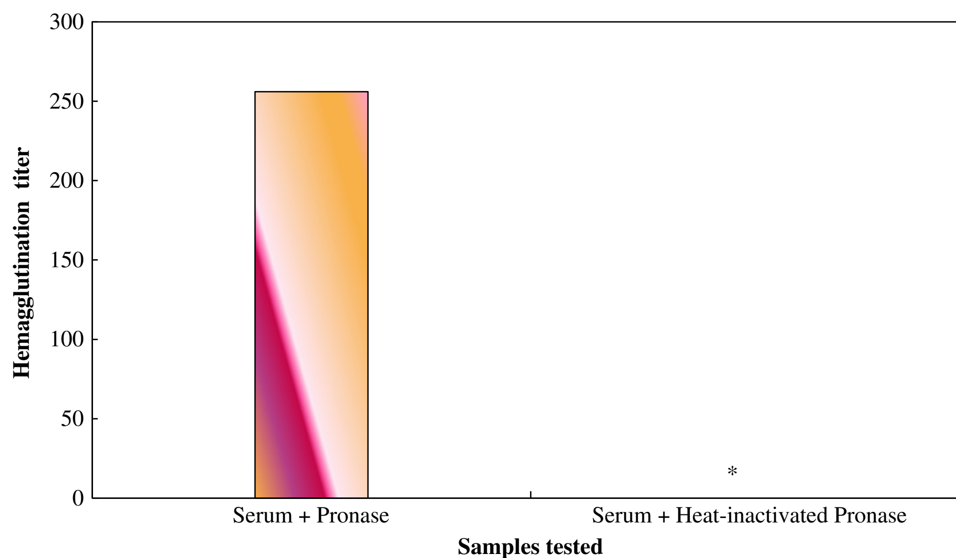

Fig. 1. Role of pronase in generation of hemagglutinating activity in serum against hen RBC. Data represent median titer values from three determinations using different stocks of pooled serum (single and multiple individuals). *No hemagglutinating activity was detectable.

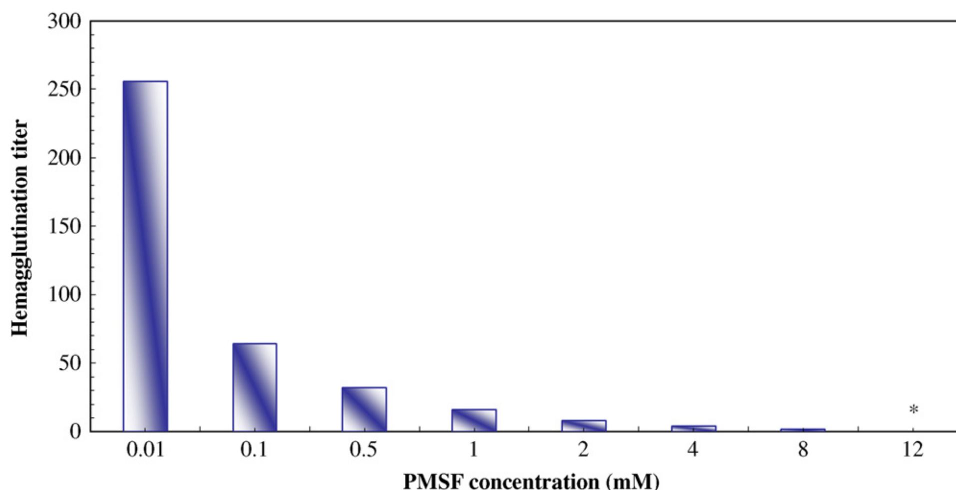

Fig. 2. Dosimetric analysis of phenylmethylsulfonyl fluoride (PMSF) on pronase inducible hemagglutinating activity in serum against hen RBC. Data represent median titer values from three determinations using different stocks of pooled serum (single and multiple individuals). *No hemagglutinating activity was detectable.

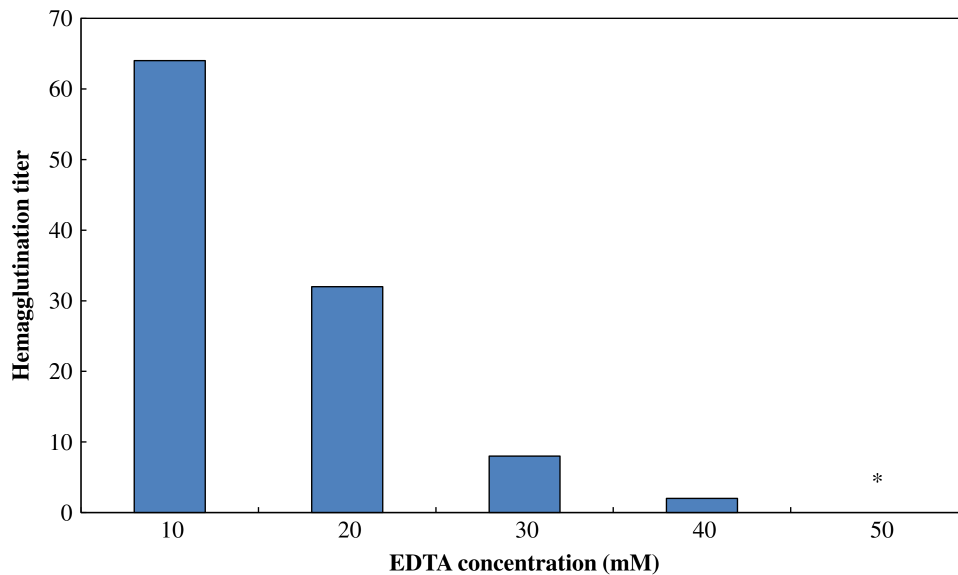

Fig. 3. Dosimetric analysis of EDTA on pronase inducible hemagglutinating activity in serum against hen RBC. Data represent median titer values from three determinations using different stocks of pooled serum (single and multiple individuals). *No hemagglutinating activity was detectable.

Table 6
Cross adsorption tests on trypsin or α-chymotrypsin-treated serum (AB).

Serum adsorbed with[a]	HA titer against RBC types tested[b]	
	Hen RBC	Sheep RBC
None	128	64
Hen RBC	0	0
Sheep RBC	0	0

[a] Three to four sequential 60 min adsorption at 28 °C.
[b] Data represent median titer values from three determinations for each RBC type using different stocks of pooled serum (single and multiple individuals).

Table 7
Time course analysis of hemagglutinating activity inducible in serum (AB) by pronase, trypsin and SDS against hen RBC.

Time intervals (min)	Hemagglutination titer against hen RBC[a]		
	Pronase-treated serum	Trypsin-treated serum	SDS-treated serum
15	256	256	128
30	256	256	128
60	256	256	128
90	256	256	128
120	256	256	128

[a] Data represent median titer values from three determinations using different stocks of pooled serum (single and multiple individuals).

hen RBC, whereas, HA titer against sheep RBC was observed only in the presence of TBS-I and not TBS-II and TBS-IV (Table 8). The pronase-treated serum gave the HA titer of 256 against hen RBC in TBS-II, -III, -IV, -VI, -VII and -VIII (Table 9).

3.8. Hemagglutination-inhibition assay

Off 53 carbohydrates tested, only eight carbohydrates were found to inhibit HA activity of untreated serum against buffalo RBC at the minimal inhibitory concentrations ranging from 12.5 to 100 mM (Table 10) and these include three glycosides, two disaccharides, two trisaccharides and one tetrasaccharide.

Only three hexosamines inhibited the HA activity of pronase treated serum against hen RBC at varying inhibitory concentrations (Table 11).

Of the 19 diverse carbohydrates the HA of trypsin-treated serum against only sheep RBC was inhibited by two *N*-acetylated aminosugars, at the minimal inhibitory concentration of 25 mM (Table 12), while, three sialoglycoproteins, inhibited the HA against both hen and sheep RBC with minimal inhibitory concentrations ranging from 0.312 to 2.50 mg/ml (Table 13). In contrast, asialo-BSM did not inhibit the activity, while, asialo-fetuin did inhibit HA against sheep RBC at a concentration of 1.25 mg/ml and was not inhibitory for agglutination of hen RBC (Table 13).

4. Discussion

Human plasma or serum is known to contain two distinct types of naturally occurring humoral molecules capable of causing agglutination of RBC. The first type includes conagglutinins represented by natural antibodies (IgM), whose reactivity is detectable with human RBC and the second type is the lectins naturally occurring in human blood. In our study, untreated serum agglutinated buffalo, rabbit, rat and mouse RBC, irrespective of their blood groups, but not ox, sheep, goat and hen RBC. This agglutination observed may be due to previously reported six lectins [14] or a new lectin, which yet requires further elucidation. In all subsequent studies, human AB serum was used as it did not possess conagglutinins.

Among five proteases tested, pronase, trypsin and α-chymotrypsin were effective elicitors of HA, while two other proteases tested, papain and pepsin were ineffective. This discrepancy in the efficiency of various proteases may be related to their distinct substrate specificity, which also appeared to be reflected by differential RBC binding property of molecules generated upon action of these proteases on native serum components. Generation of HA was also observed in serum upon treatment with only SDS, but not with Triton X-100 and Tween 20.

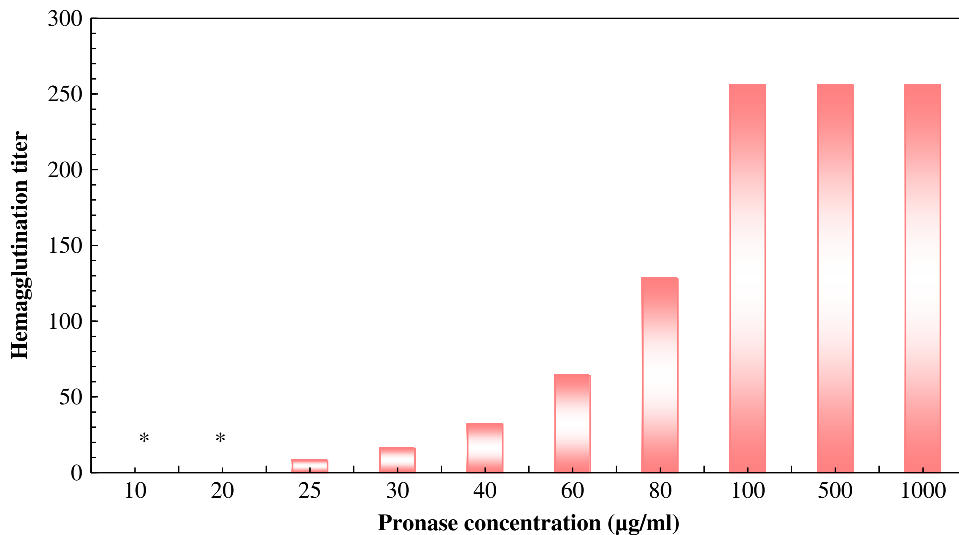

Fig. 4. Dosimetric analysis of pronase inducible hemagglutinating activity in serum against hen RBC. Data represent median titer values from four determinations using different stocks of pooled serum (single and multiple individuals). *No hemagglutinating activity was detectable.

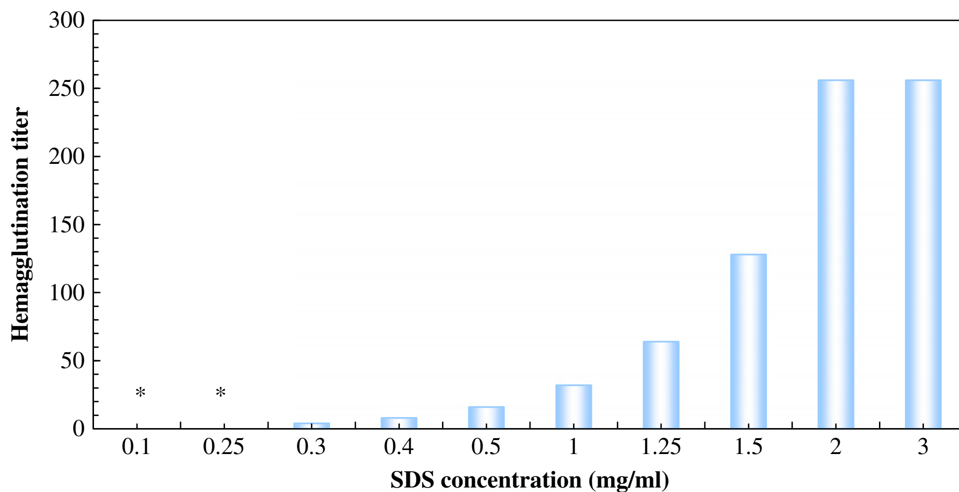

Fig. 5. Dosimetric analysis of SDS inducible hemagglutinating activity in serum against hen RBC. Data represent median titer values from four determinations using different stocks of pooled serum (single and multiple individuals). *No hemagglutinating activity was detectable.

Table 8
Cation dependency and EDTA sensitivity of hemagglutinating activity in untreated and trypsin-treated serum (AB) against various vertebrate RBC types.

Buffer types tested[a]	Serum hemagglutination titer[b]			
	Buffalo RBC[c]	Rabbit RBC[c]	Hen RBC[d]	Sheep RBC[d]
TBS-I	16	16	256	256
TBS-II	16	16	256	0
TBS-IV	8	4	256	0

[a] Each buffer was used for serial dilution of the sample as well as each RBC suspension.
[b] Data represent median titer values from three determinations for each RBC type using different stocks of pooled serum (single and multiple individuals).
[c] Target used for untreated sera.
[d] Target used for trypsin-treated sera.

Table 9
Cation dependency and EDTA sensitivity of pronase-induced hemagglutinating activity in serum (AB) against hen RBC.

Buffer types tested	Hemagglutination titer against hen RBC[a]
TBS-II	256
TBS-III	256
TBS-IV	256
TBS-VI	256
TBS-VII	256
TBS-VIII	256

[a] Data represent median titer values from three determinations using different stocks of pooled serum (single and multiple individuals).

The generation of HA in sera by pronase/SDS irrespective of their blood group identity or after recalcification of citrated whole blood or plasma, shows that certain components targeted by pronase/SDS are present in the systemic circulation and remain stable upon exposure to anticoagulant as well as recalcification process. The generation of HA is not due to direct action of these elicitors on RBC targets, since they did not agglutinate them and also the serum failed to agglutinate these targets treated with elicitors. Therefore, it becomes apparent that the exogenous elicitors generated new type of molecules by reacting only with certain serum components, whose identity will be explored. All the HA activities observed in untreated and treated sera were highly stable to storage with an

Table 10
Hemagglutination-inhibition of untreated AB serum (titer=4) by various carbohydrates against buffalo RBC.

Carbohydrates tested	Maximum concentration tested (mM)	Minimum inhibitory concentration (mM)[a]
Glycosides		
p-nitrophenyl-α-D-glucopyranoside	50	12.5
p-nitrophenyl-β-D-galactopyranoside	100	25
Methyl-α-D-mannopyranoside	200	50
Disaccharides		
Melibiose (gal α 1→6 glc)	200	25
Cellobiose (glc β 1→4 glc)	200	100
Trisaccharides		
Raffinose (gal α 1→6 glc α 1→2 fruc)	200	100
Melezitose (glc α 1→3 fruc β 2→1 glc)	200	25
Tetrasaccharide		
Stachyose (gal α 1→6 gal α 1→6 glc β 1, 2 fruc)	200	50

The following carbohydrates did not inhibit the hemagglutinating activity and unless otherwise stated, all carbohydrates were tested at a concentration up to 200 mM: *N*-acetylgalactosamine, *N*-acetylglucosamine, *N*-acetylmannosamine, *N*-acetylneuraminic acid (20 mM), β-allose, D-arabinose, L-arabinose, colominic acid (10 mg/ml), 2-deoxy-D-glucose, 2-deoxy-D-galactose, dextran-T_{70} (10 mg/ml), dextran-T_{500} (10 mg/ml), diacetylchitobiose (50 mM), D-fructose, D-fucose (100 mM), L-fucose (100 mM), galactosamine, galactose, galacturonic acid, β-gentiobiose, glucosamine, glucose, glucuronic acid, inulin (5 mg/ml), lactose, laminarin (10 mg/ml), mannan (10 mg/ml), mannosamine, mannose, maltose, maltotriose, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, methyl-β-D-glucopyranoside, *p*-nitrophenyl-α-galactopyranose (50 mM), *p*-nitrophenyl-β-D-glucopyranoside (100 mM), palatinose, raffinose, L-rhamnose, L-sorbose, sucrose, trehalose, turanose and xylose.

[a] The assay was repeated four times for each carbohydrate with identical results using different stocks of pooled serum (single and multiple individuals).

exception to the HA observed in trypsin-treated serum against sheep RBC. This observation indicated the presence of two types of activities in trypsin-treated serum: stable HA against hen RBC and an unstable HA against sheep RBC.

The inability of heat-inactivated pronase to generate HA in sera show that pronase was solely responsible for the detected activity. Inhibition of HA in pronase-treated serum by PMSF and EDTA (protease inhibitors), clearly suggests that this protease generates HA by proteolytic cleavage of certain serum components. The most potent inhibitor was PMSF because, the HA activity was completely inhibitable at a lower concentration than EDTA.

The ability of SDS to generate such agglutinin molecules may be attributed to conformational changes inflicted in various serum biomolecules [37]. Nevertheless, it is notable that in contrast to protease-treated serum the molecules induced upon SDS treatment could cross react with various human RBC types, suggesting differential characteristics.

Cross adsorption tests were performed to assess RBC binding specificity of newly generated agglutinin molecules where, these two RBC types equivocally adsorbed the agglutinating activity from the sera treated with trypsin or α-chymotrypsin, thereby suggesting that these agglutinin molecules are capable of reacting with both hen and sheep RBC.

Time course analysis revealed that pronase, trypsin or SDS could rapidly generate HA. Dosimetric response in HA generation was observed with both pronase and SDS, wherein, pronase was most efficient to generate HA at a low concentration of 100 μg/ml, because it is a mixture of several proteases and non-specific in action [38].

Table 11
Hemagglutination-inhibition of pronase-treated AB serum (titer=4) by various carbohydrates, amino acids and a phospholipid against hen RBC.

Carbohydrates tested	Maximum concentration tested (mM)	Minimum inhibitory concentration (mM)[a]
Hexoses		
Glucose	200	–
Galactose	200	–
Mannose	200	–
Hexosamines		
Glucosamine	200	50
Galactosamine	200	100
Mannosamine	200	25
***N*-acetyl hexosamines**		
N-acetylglucosamine	200	–
N-acetylgalactosamine	200	–
N-acetylmannosamine	200	–

The following carbohydrates did not inhibit the HA activity and unless otherwise stated, all carbohydrates were tested at concentration up to 200 mM: *N*-acetylneuraminic acid (100 mM), β-D-allose, D-arabinose, L-arabinose, cellobiose, colominic acid (10 mg/ml), 2-deoxy-D-galactose, 2-deoxy-D-glucose, dextran T_{70} (10 mg/ml), dextran T_{500} (10 mg/ml), D-fructose, D-fucose (100 mM), L-fucose (100 mM), D-galacturonic acid, gentiobiose, D-glucuronic acid, inulin (5 mg/ml), lactose, laminarin (10 mg/ml), maltose, maltotriose, mannan (10 mg/ml), mannose, melezitose, melibiose, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, methyl-α-D-glucopyranoside, methyl-β-D-glucopyranoside, methyl-α-D-mannopyranoside, *p*-nitrophenyl-α-D-galactopyranoside (50 mM), *p*-nitrophenyl-β-D-galactopyranoside (100 mM), *p*-nitrophenyl-α-D-glucopyranoside (50 mM), *p*-nitrophenyl-β-D-glucopyranoside (100 mM), palatinose, raffinose, L-rhamnose, ribose, L-sorbose, stachyose, sucrose, turanose, trehalose and D-xylose.The following amino acids also did not inhibit the HA activity at concentration tested up to 200 mM: *N*-acetylglutamine, *N*-acetylglycine, *N*-acetylhydroxyproline, O-acetylhydroxyproline, L-Alanine, L-Arginine monochloride, L-Cystine, L-Cysteine.HCl, L-Glutamic acid, Glutamine, Glycine, L-Histidine, L-Hydroxyproline, Isoleucine, L-Lysine.HCl, L-Methionine, DL-Nor-Leucine, Ornithine.HCl, Phenylalanine, L-Proline, DL-Serine, DL-Threonine, DL-Tryptophan, L-Tyrosine and DL-Valine Phosphoryl choline (a phospholipid) tested up to a concentration of 200 mM was non-inhibitory.

– Non-inhibitory.

[a] The assay was repeated four times for each test substance with identical results using different stocks of pooled serum (single and multiple individuals).

Table 12
Hemagglutination-inhibition of trypsin-treated AB serum (titer=4) by various carbohydrates and a phospholipid against hen and sheep RBC.

Carbohydrates tested	Maximum concentration tested (mM)	Minimum inhibitory concentration (mM)[a]	
		Hen RBC	Sheep RBC
Hexoses			
Glucose	200	–	–
Galactose	200	–	–
Mannose	200	–	–
Hexosamines			
Glucosamine	200	–	–
Galactosamine	200	–	–
Mannosamine	200	–	–
***N*-acetyl hexosamines**			
N-acetylglucosamine	200	–	–
N-acetylgalactosamine	200	–	–
N-acetylmannosamine	200	–	25
Sialic acid			
N-acetylneuraminic acid	100	–	25

The following carbohydrates did not inhibit the hemagglutinating activity against both hen and sheep RBC and unless otherwise stated, all test substances were tested at a concentration up to 200 mM: arabinose, colominic acid (10 mg/ml), dextran T_{70} (10 mg/ml), D-fructose, laminarin (10 mg/ml), mannan (10 mg/ml), ribose, sucrose and xylose. Phosphoryl choline (a phospholipid) tested up to a concentration of 200 mM was non-inhibitory.

– Non-inhibitory.

[a] The assay was repeated three times for each test substance with identical results using different stocks of pooled serum (single and multiple individuals).

Table 13
Hemagglutination-inhibition of trypsin-treated AB serum (titer=4) by glycoproteins against hen and sheep RBC.

Glycoproteins tested	Maximum concentration tested (mg/ml)	Minimum inhibitory concentration (mg/ml)[a]	
		Hen RBC	Sheep RBC
Bovine submaxillary mucin (BSM)	10	1.25	0.312
Asialo-BSM	5	–	–
Fetuin	10	2.50	2.50
Asialo-fetuin	5	–	1.25
Porcine thyroglobulin	10	5	0.625

– Non-inhibitory.

[a] The assay was repeated three times for each glycoprotein with identical results using different stocks of pooled serum (single and multiple individuals).

Most of the naturally occurring serum lectins were cation dependent and sensitive to EDTA, but in our study all the observed HA were cation independent and insensitive to EDTA suggesting that new type of molecules has been detected and it warrants more investigation in detail. However, trypsin-treated serum showed once again a variation, wherein, the HA against sheep RBC was cation dependent and EDTA sensitive. This result further confirms the presence of two types of HA molecules in trypsin-treated serum.

HA of untreated serum was inhibitable by wide range of carbohydrates such as glycoside derivatives with *p*-nitrophenyl or methyl group, di- or oligo-saccharides containing terminal glucose or galactose with varying glycosidic linkages and this profile was not overlapping with any of the specificities reported for the naturally occurring serum lectins. Furthermore, it was not comparable too as to whether the HA observed was due to reported lectins or some new molecules since some carbohydrates were not tested in previous reports. Thus, it is likely that the lectins reported previously in serum or some new type of molecules yet to be explored may be responsible for the HA. Interestingly, HA of pronase-treated serum was inhibited only by the three hexosamines indicating generation of *anew* type of lectin molecules, termed neo-lectin with distinct specificity varying from that of natural lectins reported by other investigators and us. In trypsin-treated serum, different inhibitors could inhibit HA against hen and sheep RBC implicating generation of two kinds of neo-lectin molecules that differ in ligand binding specificities.

5. Conclusion

Overall, to conclude, the findings of this study clearly demonstrate, *for the very first time*, a complete HA profile for untreated serum against various vertebrate RBC types and the possibility for generation of lectin molecules in human serum by exogenous elicitors. Besides, these neo-lectin molecules generated by proteases implicated restriction in their self-reactivity (with human RBC types) and thus it is envisaged that they might potentiate immuno-defense process *in vivo* against foreign invaders.

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