

Original Article

A novel biologic immunomodulator, HDFx, protects against lethal hemorrhage, endotoxins and traumatic injury: potential relevance to emerging diseases

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Abstract: For more than 125 years, it has been known that the RES, macrophages and the innate immune system play fundamental roles in host defense against pathogenic infections, trauma, hemorrhage, and combined injuries. Some years ago, we and others reported that the RES-macrophage system was intimately connected to resistance to these bodily stressors, among other injuries. We tested the hypothesis that induction of tolerance (either spontaneous, RES-stimulated, or drug-induced) might be associated with production of a yet-to-be-identified biologic host defense factor, which we have termed HDFx. The results presented, herein, demonstrate for the first time that: 1) the MW of this protein, HDFx, is approximately 35-40 KDa, larger than known defensin peptides and much smaller than the larger MW fibronectins and complement products; 2) we describe some of HDFx's physico-chemical characteristics; 3) approximately 80 % of HDFx's plasma biological activity is derived from macrophages; 4) about 15-20 % of its activity is derived from natural killer (NK) cells; 5) polymorphonuclear leukocytes are not a source of HDFx synthesis or release; 6) known stimulants of the RES-macrophage system (i.e., denatured human serum albumin, triolein, and choline chloride) effect phagocytic stimulation of macrophages and protection against endotoxins, trauma, and hemorrhage via synthesis and release of HDFx; 7) adaptation to lethal trauma is dependent on the biological activity of HDFx; and 8) repeated administration of purified HDFx to rats, over several months, does not produce any detectable pathologies. Lastly, the release of cytokines (i.e., IL-2, IL-6, IFN-gamma) from lymphocytes, after hemorrhage and trauma, at least in rodents, appears to be dependent on the available plasma levels of HDFx. Since it is present also in mice, guinea-pigs, and rabbits, we are tempted to speculate that HDFx could prove (if found in humans) to be useful against potential biothreats, new emerging diseases, high-risk surgical procedures, hospital-borne infections, and burn injuries, where the chances for superimposed bacterial infections present great risk.

Key words: Macrophages, reticuloendothelial system, natural killer cells, leukocytes, phagocytes, lymphocyte cytokines

Introduction

In the next two decades, the civilian and military populations of the U.S.A. may be faced with an array of biothreats and infectious microorganisms, some emerging from the environment with others generated by bioterrorists. Vaccines, antibiotics and antiviral drugs will all be useful in protecting against many of these threats but cannot be counted upon in the event of rapid assaults from genetically-altered, mutated or drug-resistant microbes. Approximately 4,000 people have

died worldwide from the H1N1-mutated flu virus with no end in sight. Added to this, are the approximately two million cases of hospital-borne (nosocomial) infections with approximately 100,000 deaths per year in the U.S.A., alone. These patients, unfortunately, eventuate in a compromised immune system, particularly a loss of many innate immune functions [1-3]. A new disturbing trend in antimicrobial resistance of gram-negative pathogens and "superbugs" has seriously complicated the treatment of these patients [1, 2, 4-6]. In addition, our country, over the

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past several years, has been faced with numerous deaths from contaminated (with bacteria) meats, vegetables, and seafoods [7-9]. Many of the emerging diseases such as the H1N1 flu, SARS, dengue fevers, etc., have a very serious hemorrhagic component to them which complicates effective treatment [10, 11]. Our governmental resources seem to remain powerless to combat these assaults on our populations.

Each year, in the U.S.A., alone, more than 150 million prescriptions are written, 60% of which are for antibiotics. Of these, it has been estimated that 50 million of these costly prescriptions are probably unnecessary [12]. Added to this, is the ever-growing and soaring worldwide use of antibiotics in agriculture. How much is this indiscriminate use of antibiotics contributing to the ever-growing resistance of pathogens to antibiotics noted above?

Ever since the classic work of the Noble Prize-winning laureate, Elie Metchnikoff, more than 125 years ago [13], the important contributions of macrophages and phagocytic leukocytes to natural (innate) resistance against pathogenic bacteria and viruses have been recognized. Over the past three-four decades, considerable evidence (from both animals and humans) has accrued to support a strong relationship of the functional (physiological) state of macrophages-phagocytes and natural killer (NK) cells to host defense and resistance to pathogens, hemorrhage, trauma, surgery, burns, anaphylaxis, parasitic infections, and wound healing [for reviews, see 14-22]. NK cells are extremely versatile and are pivotal in killing tumor cells, virus-infected cells, parasites and fungi [22, 23]. However, it is still not clear as to what signals/trigger activation of these important NK cells [22, 23]. The cells of the reticuloendothelial system (RES, also termed the mononuclear-phagocytic system) are major participants in the innate immune response and possess numerous pathophysiological functions: 1) phagocytosis of bacteria; 2) production of inflammatory mediators; 3) synthesis of molecules affecting antibacterial defense, blood clotting, cell growth, vascular growth, tumor growth, and collagen production; 4) initiation of the immune response; 5) clean-up operations (scavenger activity); and 6) induction of general effects such as angiogenesis, hematopoiesis, fever, acute phase

reaction of the inflammatory response, among other important bodily functions [14-22,24].

Our laboratories, over several decades, as well as others, have reported that: 1) mammals (including humans) subjected to various forms of lethal infections, hemorrhage, trauma, as well as combined injuries exhibit quantitative, sequential changes in RES cell activities i.e., the greater the insult, the greater the prolonged depression in phagocytic activities [25- 31]; 2) mammals (including humans) which spontaneously recover (or are on the road to recovery after appropriate therapies) from pathogens, circulatory shock, hemorrhage, trauma, radiation, and severe injuries exhibit increased RES activities [25-30]; 3) animals which spontaneously recover fully on their own, from severe systemic infections or trauma-shock, exhibit a cross-tolerance to other forms of circulatory shock and trauma and do not show any signs of depression or compromise of their immune systems [14, 15, 17, 26, 27, 30, 31]. In addition, we and others have reported that certain stimulants of the RES-mononuclear phagocytic system, compatible for human use, produce a tolerance to the lethal effects of bacteria, hemorrhage, trauma, irradiation, and combined injuries [14, 15, 32- 37]. We hypothesized that induction of tolerance (either spontaneous, RES - stimulated, or drug-induced) might be associated with production of a yet-to-be-identified biologic host defense factor, which we have termed HDFx.

The results reported, herein, serve to demonstrate, for the first time, many of the attributes of this newly-discovered biologic immunomodulator, including: 1) its approximate molecular weight, 2) some of its physicochemical characteristics, 3) what cells most likely are the major source of its production in-vivo, 4) how its production can be stimulated in-vivo, 5) the relative potency of various pathophysiological stimuli to manufacture, in vivo, sizable quantities of HDFx, and 6) some unique effects on the immune system.

Materials and methods

Animals, anesthesia, and survival studies

Young, adult inbred male Wistar strain rats (165-200g) were used for all studies. All animals were anesthetized prior to surgery, hemorrhage, endotoxins, trauma, combined

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injury, and blood withdrawal with pentobarbital sodium (Nembutal, 4 mg/100 g i.m.). To reduce genetic variability as a factor [14, 38], littermates from our breeding colony were used throughout these studies. All animals were at least 70 days old and, thus, sexually mature [39]. Only male animals were included as the sex of animals has been demonstrated by a number of workers to influence the outcome of shock-trauma studies and the responses of macrophages to physiologic and pharmacologic stimuli [14, 19, 37, 40]. All animals were given sterilized distilled water to drink and Purina rat chow *ad libitum*. Each study group (controls and experimentals) were comprised of at least 12 rats. Each anesthetized animal, prior to surgery or animal model procedure, was placed on a temperature-controlled table and maintained under temperature-controlled conditions. All animals subjected to hemorrhage, trauma, endotoxins, and combined injury were carefully monitored for survival for at least 7 days. If any animal appeared to demonstrate any pain or other untoward effects, it was sacrificed with a lethal dose of pentobarbital sodium (i.e., 100 mg/100 g). This investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised in 1996) and was approved by the *Animal Use and Care Committee at SUNY Downstate Medical Center*.

Hemorrhage models

The procedure for normal animals followed closely methods we have described previously and was done under as close as possible to sterile conditions [29]. Briefly, a femoral artery and vein were cannulated in each animal. Both catheters contained heparinized sterile, isotonic saline. The femoral artery was connected to a bleed-out reinfusion device containing heparinized sterile, isotonic saline and in tandem with a conventional, calibrated mercury manometer. Following initial control observations, over a period of 15-20 min, the animal was bled in graded fashion over a 20-30 min period to a mean blood pressure level of 30-35 mm Hg and maintained at this level for 120 min by additional small bleedings as necessary [29, 41]. No blood was reinfused during these hypotensive periods after which 2.0 ml of normal, isotonic sterile saline was infused intravenously at a constant rate for 60 min. At the conclusion of this infusion the shed

blood was reinfused (intra-arterially) over a 20-30 min period [29, 41]. This procedure resulted in a 60-70 % mortality [29]. Different animals were then either: 1) observed and carefully monitored for survival, 2) subjected to tests for RES phagocytic indices at various intervals of time, i.e., 120 min and 24 hr (see below), 3) subjected to blood withdrawal at 24 hr, in survivors, for cytokine and interferon-gamma levels, or 4) subjected to intracarotid arterial blood removal for isolation of HDFx at 24hr of survival (see below).

Endotoxin models

The procedure for normal, anesthetized animals closely followed what we have described previously [33, 35]. All experiments were carried out as close to aseptic conditions as possible. Each animal was placed on a temperature-controlled table. Briefly, we inserted sterile heparinized catheters in the carotid artery (for blood pressure measurement and withdrawal of blood) and femoral vein for iv injection of either normal, sterile isotonic saline (controls) or endotoxins (either *E. coli* [LPS serotype 055:B5, Difco, Detroit, MI] or *Salmonella enteritidis* [Difco No.12047, Detroit, MI], 2.0 mg/kg). This dose of endotoxins resulted in a 55-70 % mortality [33, 35]. After a 30 min period of stabilization, baseline blood pressure was recorded and then either the saline vehicle or a single dose of endotoxin was injected over a 2-min period into the femoral vein. Four hr later, bloods were drawn via the carotid artery for phagocytic indices (see below). 24 hr later, bloods were drawn in all survivors for phagocytic indices and isolation of HDFx (see below). These two gram-negative endotoxins were chosen for two major reasons: 1) they have both been found in the past few years to be the major contaminants in different foods and vegetables and to be responsible for a number of deaths in the U.S.A., and 2) *S. enteritidis* has been demonstrated by the Pasteur institute to faithfully mimic human typhoid [42].

Traumatic shock model and adaptation to trauma

For these studies, we subjected different groups of anesthetized animals to whole-body trauma using Noble-Collip drum trauma, as we have described previously [26]. This type of shock-trauma was utilized as it can be readily standardized and is relatively amenable to

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quantitation of the imposed stress [26]. All experiments were carried out under as close to aseptic conditions as possible. We subjected the different groups of animals to 400,600, or 850 revolutions of Noble-Collip trauma [26]. Bloods were drawn at 2 hr, and in all survivors, at 48 hr for: 1) phagocytic indices (see below), 2) isolation of HDFx, and 3) cytokine and interferon-gamma levels (see below). A 48 hr time period was chosen based on previous studies from our laboratories which demonstrated that this time frame resulted, in survivors, in peak hyperstimulation of the RES [26]. Buprenorphine (50 µg/kg) was given every 8 hr during the post-trauma period.

If hyperphagocytic and immune functions are, indeed, related to increased survival after experimental shock-trauma, then animals exhibiting hyperphagocytic-immune functions should demonstrate elevated levels of HDFx. In order to test this hypothesis, we subjected anesthetized animals, which were previously subjected to 400 revolutions of non-lethal trauma, a stress-intensity known to induce hyperphagocytosis [26], to 850 drum revolutions which would, in itself, result in an 80-100 % mortality in untraumatized control rats [26]. Bloods were drawn after 48 hr for: 1) phagocytic indices, and 2) levels of HDFx.

RES phagocytic function

The procedure in these experiments essentially consisted of determining RES phagocytic indices (K values) [26, 29] in the different groups of experimental animals (above) at the stipulated intervals of time. The phagocytic indices were determined by measuring the rate of clearance of colloidal carbon (either 4 or 8 mg in calf skin gelatin/100 g body wt) [26, 29]. Precisely timed blood samples were obtained at 2, 4, 8, 12, and 15 min after the *iv* colloidal carbon injection, hemolyzed in 0.1 % sodium carbonate and the carbon concentrations measured photometrically at 675 mu [26, 29]. The colloidal carbon utilized in these studies was Pelikan C11/1431 (Gunther-Wagner, Hannover, Germany). Phagocytic indices were calculated:

$$K = \log_{10} C_1 - \log_{10} C_2 / t_1 - t_2$$

where K is the phagocytic index and C₁ and C₂ are the colloidal carbon concentrations in mg/100 ml of blood at t₁ and t₂ [14, 26, 29].

Effects of RES stimulants on survival after hemorrhage, endotoxins, trauma, phagocytic indices and on HDFx levels

Three types of materials were utilized based on previous studies: 1) mildly denatured human serum albumin aggregate (10-20 mu) as prepared for liver scans (Squibb Labs, NJ), but untagged [33]; 2) highly purified choline chloride (Sigma, St. Louis, MO) [32]; and 3) triolein (glycerol trioleate) emulsified in 0.5 % Tween-20 (Sigma, St. Louis, MO) in 5 % dextrose (highly purified, Fisher Scientific, Pittsburgh, PA), homogenized at 19,000 rpm [35]. Pretreatment-dose-frequency-duration regimens were worked out in rats for each of the above, such that maximum RES clearance rates were obtained 24 hr following the last dose: 1) albumin, 21.6 mg/kg *iv* twice daily for three consecutive days; 2) choline, 20 mg/kg, *i.m.*, twice daily for three consecutive days; and 3) triolein, 250 mg/kg, *iv*, once daily for two consecutive days.

Effects of hemorrhage and trauma on cytokine levels in lymphocytes with/without pretreatment with HDFx

Lymphocytes were harvested from control animals, hemorrhaged survivors, and survivor animals subjected to whole-body trauma according to previously-described routine density-gradient methods utilizing a typical Metrizoate-Ficoll mixture [43]. The lymphocytes were then analyzed for levels of IL-2, IL-6, and interferon -gamma (IFN-gamma) using commercially available antibody-kits (Santa Cruz Labs, Santa Cruz, CA). Different groups of animals received either injections (*i.v.*) of sterile, isotonic saline (vehicle) or HDFx (4 injections over 48 hr, see Methods below) prior to harvesting the lymphocytes.

Isolation of HDFx from pooled blood samples

Briefly, heparinized blood was removed from the experimental and control animals by either carotid or aortic arterial cannulation. To isolate HDFx, pooled plasma from naïve (control), stimulated, or survival animals was brought to pH 5.5 by addition of 1 N HCl, heated at 60° C for 10 min and filtered using a series of Millipore filters (Ultrafiltration Discs, 10-100 kDa; Millipore Corp., Billerica, MA). These gradient filtration-disc pore sizes were also used to get an estimate of the MW of HDFx and to determine if HDFx was non-dialyzable.

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Plasma HDFx isolates were stored frozen at -20°C or less until use.

Use of Limulus Amebocyte lysate (LAL) and precautions taken for blood collections

Pooled plasmas to be tested for HDFx biological activity were routinely screened for gram-negative endotoxin using a LAL assay [44,45]. All our studies employed depyrogenated blood extraction tubes; all glassware was first wrapped in aluminum foil and baked at 240°C for 4 hr. All blood collection tubes were certified to be endotoxin-free. Any gloves worn during blood handling, collection, and needed for performing LAL assays were powder-free (most powders contain endotoxin). The reference standard endotoxin (RSE) we used was made from *E. coli* O113 (known as EC-6). Any endotoxin detected was recorded as endotoxin units (one EU = 100 pg RSE) and reported as EU/ml.

Bioassay and standardization of HDFx

We arbitrarily set one unit of purified HDFx (i.e., 0.25 ml x 4 injections) administered over 48 hr s.c. or i.v. to be equivalent to raising the RES phagocytic index 100% in naïve rats, 12-24 hr later. Two units of HDFx (i.e., a total of 2.0 ml or 8 injections s.c. or i.v.) over 48 hr raised the RES phagocytic index 200% over control levels.

Determination of which subsets of cells are needed for HDFx production/induction of resistance

To determine whether production/induction of resistance required a specific subset of leukocytes and/or macrophages, we performed specific depletions. NK cells or polymorphonuclear leukocytes (PMNs) were individually depleted with injections of anti-NK (PharMingen, San Diego, CA—three i.p. injections of 500 µg each, at 2-day intervals) [46] or anti-Ly6G (1:25, BD Biosciences, San Diego, CA—three i.p. injections of 500 µg each, at 2-day intervals) [47], respectively. Since, at the time of our studies, no specific depleting antibodies were available for macrophages, we utilized an injection of 2-chloroadenosine (25mg/kg, injected ip) [48]. These depletions did not affect unrelated leukocyte cell populations (unpublished data).

Blockade of the RES-macrophage system with colloidal substances

It has been demonstrated, repeatedly, by many investigators that certain colloidal substances can either attenuate greatly, or completely, block the ability of RES macrophages to phagocytize particulate matter and live microorganisms [14, 15, 17, 49, 50]. Among the colloids that induce this blockade are colloidal carbon, gadolinium chloride, Thorotrast (a stabilized colloidal suspension of thorium dioxide), and Proferrin (a saccharated iron oxide). Using either a colloidal carbon preparation stabilized in gelatin (see above, 64 mg/100 g bw, Pelikan Werke, Germany), Thorotrast (400 mg/kg bw), or Proferrin (80 mg/kg), given i.v. to different groups of rats, we subjected the anesthetized animals to repeated drum trauma adaptation procedures (see above) or to iv administered LD₅₀₋₆₀ doses of *S. enteritidis* endotoxin (see above, 2.0 mg/100 g bw). Survival and levels of HDFx were measured at 24 hr after adaptation.

Statistical analyses

Where appropriate, means and means +/- S.E. were calculated. Differences between means were assessed for statistical significance by Student's t-tests and ANOVA, followed by a Newman-Keuls test. Mortality/survival differences were tested for statistical significance by Chi-square. Where appropriate, linear regression analysis and correlation coefficients were determined. A P value < 0.05 was considered significant.

Results

RES phagocytic indices early after injury and in survivors after hemorrhage, endotoxins, trauma and adaptation to lethal trauma

Table 1 indicates that irrespective of systemic circulatory stress (i.e., hemorrhage, endotoxins, or trauma), the RES phagocytic indices are severely and significantly depressed, similar to that reported previously [14, 18, 25, 26, 28, 29]. However, the results shown in **Table 1** indicate that within 24-72 on survival, these severely stressed rats all exhibit hyperfunctional abilities to phagocytose particulate matter. Although not shown, all those animals that go on to die from these stresses continue to exhibit severely compromised

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Table 1. RES phagocytic function early after injury and in survivors after hemorrhage, endotoxins, and trauma and adaptation to lethal trauma

Time after Trauma (hrs)	Hemorrhage	Endotoxin	Trauma	Adaptation
Controls	0.045+/- 0.003	0.046+/- 0.004	0.048+/- 0.004	0.045+/-0.003
1-3	0.015+/-0.002*	0.010+/-0.003*	0.010+/-0.003*	0.075+/-0.006*
48	0.090+/-0.005*	0.080+/-0.008*	0.120+/-0.006*	0.165+/-0.010*

N=16-24 animals /group. Controls =60 animals. Values =means +/-S.E.M.

*P<0.001 compared to controls. Test colloidal carbon dose = 4mg/100 g bw.

Table 2. Adaptation to lethal traumatic shock results in hyperstimulation of the RES phagocytic function

Time After Adaptation (hr)	Phagocytic Index
Controls	0.050+/-0.004
24	0.095+/-0.005*
48	0.200+/-0.012*
72	0.098+/-0.008*
144	0.047+/-0.003

Controls =32 animals. Experimentals = 16-24 animals/group.

*P<0.001 compared to controls.

phagocytic systems until death, as shown previously [14, 26, 28, 29].

Adaptation to lethal traumatic shock results in a vast hyperstimulation of RES phagocytic function

In order to determine whether there is a close correlation between the capacity of the phagocytic cells of the RES and tolerance to trauma, as suggested by our previous studies and strongly reinforced by work of others [17, 19, 30, 31, 34], we exposed different groups of paired anesthetized animals to a series of repeated traumatic episodes (see Methods, and ref 26), to learn whether the phagocytic indices correlated with susceptibility or tolerance to subsequent lethal episodes of trauma. As can be seen from the data in **Table 2**, at 24-48 hr, the rats not only exhibit RES phagocytic indices which are 125-300% stimulated over naïve controls but are completely resistant to lethal trauma. In fact, although not shown, these adapted animals are often resistant to LD₂₀₀₋₃₀₀ bouts of trauma. At 72 hr, there is a slight fall in the degree of hyperstimulation with some mortality, whereas at 144 hr, there is a marked fall in the RES hyperstimulation to control levels with a return to control 80-100

% mortality. Using linear regression analyses, there is a very good correlation between the RES phagocytic index and protection against lethal trauma; the greater the degree of RES stimulation, the greater the resistance to trauma and vice-versa ($r = 0.87-0.92$, $p < 0.0$).

Diverse RES stimulants induce protection against lethal stresses

The data shown in **Tables 3-5** indicate that denatured human serum aggregate albumin, triolein and choline chloride all induce hyperstimulation of the RES phagocytic cells, but to different degrees, as reported previously [32, 33, 35]. Interestingly, the survival data (**Tables 3-5**) suggest that there is a clear variability in the ability of the stimulants to induce protection against hemorrhage, endotoxins and trauma, with albumin = triolein >> choline. A closer examination of these data reveal that the greater the RES stimulation, the greater the protection ($r = 0.82$, $p < 0.01$).

Isolation of HDFx from survivors, trauma-adapted, and stimulated animals

Using the techniques and precautions outlined in the Material and Methods, we determined that the MW of HDFx is approximately 35-40

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Table 3. Effects of denatured human aggregate albumin on RES phagocytic indices and survival after hemorrhage, endotoxin, and trauma in rats

Group	Phagocytic Index	Survivors/Total Rats	% Survival
Naive Controls	0.025+/-0.002	30/30	100
Saline Controls	0.020+/-0.003	16/16	100
Hemorrhage (saline)	0.010+/- 0.002*	12/36 *	33*
Trauma (saline)	0.005+/-0.001*	4/24*	17*
Albumin Alone	0.072+/-0.012**	16/16	100
Endotoxin (saline)	0.008+/-0.003*	10/22*	45*
Hem +Albumin	0.050+/-0.008**	20/30**	66*
Trauma +Albumin	0.042+/-0.006**	10/20* *	50*
Endotoxin+Album	0.035+/-0.005**	36/38**	95*

Data for phagocytic indices are means +/- S.E.M. Test dose colloidal carbon = 8 mg/100 g bw. Blood samples were withdrawn 3 hr after hemorrhage trauma, or *S. enteritis endotoxin*.

*P<0.01 compared to naïve controls and saline controls.

**P<0.01 compared to paired hemorrhage, trauma, or endotoxin controls given only saline.

Table 4. Effects of triolein on RES phagocytic indices and survival after hemorrhage, endotoxin, and trauma

Group	Phagocytic index	Survivors/Total Rats	% Survival
Naïve Controls	0.023+/-0.003	17/17	100
Saline Controls	0.025+/-0.002	18/18	100
Hemorrhage (Tween 20/dextrose)	0.009+/-0.003*	8/26*	31*
Trauma (Tween20/dex)	0.004+/- 0.002*	3/20*	15*
Endotoxin(Tween20/dex)	0.010+/-0.002*	10/28*	36*
Triolein(Tween20/dex)	0.135+/- 0.025*	18/18	100
Hem+ Triolein	0.080+/-0.008**	16/20**	80**
Trauma +Triolein	0.065+/-0.006**	12/20**	60**
Endotoxin+Triolein	0.050+/-0.008**	14/20**	70**

Data for phagocytic indices are means +/- S.E.M. Test dose colloidal carbon =8 mg/100 g bw. Bloods were withdrawn for phagocytic indices 3 hr after hemorrhage, trauma, or *S. enteritidis* endotoxin.

*P<0.01 compared to naïve controls and saline controls.

**P<0.01 compared to paired hemorrhage, trauma, or endotoxin controls given Tween 20-dextrose diluents(vehicle) for triolein.

KDa. It is non-dialyzable. It does not contain any endotoxins (tested by the LAL assay). Whole plasma obtained from control rats does not confer any protection against the animal models used herein (more than 100 animals were tested; data not shown).

Table 6 demonstrates that isolation of plasma (filtration, and processing for HDFx-see Methods) from control, naïve animals denotes an inability to stimulate RES phagocytic function when injected into control rats. However, when plasma is processed from animals which survived hemorrhage, endotoxin injections, or trauma (after 24-48 hr),

and then injected iv (i.e., one unit=4x 0.25 ml injections of HDFx) into naïve rats, there is clear stimulation of RES phagocytic function, which is in marked contrast to plasma extracts obtained from these stressed animals, early (2-4 hr) after blood loss, endotoxins, or trauma. Our results also demonstrate that adaptation to lethal trauma induces levels of HDFx (in one unit) which are 4-5-fold over that obtained in singly-stressed survivors.

Table 7 serves to demonstrate that pretreatment of rats with the three different RES stimulants (albumin, triolein, or choline) induce different levels of HDFx in the treated

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Table 5. Effects of choline chloride on RES phagocytic indices and survival after hemorrhage, endotoxin, and trauma

Group	Phagocytic index	Survivors/Total	% Survival
Naïve Controls	0.025+/-0.002	20/20	100
Saline Controls	0.023+/-0.003	19/19	100
Hemorrhage (saline)	0.010+/-0.002*	10/28*	36*
Trauma (saline)	0.002+/-0.001*	2/20*	10*
Endotoxin (saline)	0.008+/-0.002*	8/26*	31*
Hem + Choline	0.035+/-0.004**	19/30**	63**
Trauma + Choline	0.008+/-0.003*	2/24*	8*
Endotoxin + Choline	0.010+/-0.004*	8/28*	29*

Data for phagocytic indices are means +/- S.E.M. Test dose colloidal carbon = 8 mg/100 g bw. Bloods were withdrawn 3 hr after hemorrhage, trauma, or *S. enteritidis* endotoxin.

*P<0.01 compared to naïve controls and saline controls.

**P<0.01 compared to paired hemorrhage, trauma, or endotoxin controls given saline.

Table 6. Plasma HDFx activities obtained from survivor rats after hemorrhage, trauma, and endotoxins, and after adaptation to lethal trauma as assessed by phagocytic capability

Group	Phagocytic Index
Saline Controls	0.025+/-0.002
Hemorrhage	0.060+/-0.008*
Trauma	0.075+/-0.008*
<i>S. enteritidis</i> endotoxin	0.058+/-0.006*
<i>E. coli</i> endotoxin	0.062+/-0.008*
Trauma Adaptation	0.150+/-0.016**

Data are means +/- S.E.M. HDFx was isolated from shock-trauma survivors or after adaptation to lethal trauma (see Methods) and represent phagocytic indices obtained in naïve rats given 4 injections (of the purified extract, over 48 hr). Phagocytic indices were determined on the 3rd day using 8 mg/100 bw colloidal carbon.

*P<0.01 compared to controls.

**P<0.001 compared to all other groups (ANOVA).

animals, and exhibit a relative potency, where triolein > albumin >>> choline (ANOVA, p<0.05).

It is important to point out, here, that plasma extracts from non-stimulated or non-stressed (non-shocked) animals do not, significantly, increase RES phagocytosis or alter cytokine levels in lymphocytes (see below).

Effect of hemorrhage and trauma with/without HDFx pretreatment on cytokine levels in lymphocytes

When a host is overcome by various systemic injuries, there normally is a loss in ability of lymphocytes to produce a variety of cytokines (e.g., IL-2, IL-6, IFN-gamma, etc.) [17, 19]. We hypothesized that HDFx might protect lymphocytes, allowing them to produce their usual repertoire of cytokines despite the presence of

injury. The results shown in **Table 8** indicate that, as expected, early (1-4 hr) after hemorrhage or trauma, lymphocytes harvested from the blood of these injured rats showed profound deficits (70-90 % losses) in levels of IL-2, IL-6 and IFN-gamma. However, lymphocytes obtained from animals pretreated (8 doses over 48 hr) with HDFx (obtained from trauma-adapted animals) were able to manufacture these three cytokines at levels comparable to those in non-hemorrhaged and non-traumatized rats (**Table 8**).

Macrophages and NK cells required for manufacture of HDFx and resistance

To determine whether a specific subset of leukocytes and/or macrophages is required for adaptation to trauma and manufacture of HDFx, we utilized specific depletions. As stated

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Table 7. Effects of RES stimulants on plasma biological activities (phagocytic indices) of HDFx when given to naïve rats

RES Stimulant	Phagocytic Index
Saline Controls	0.023+/-0.003
Choline Chloride	0.040+/-0.005*
Aggregate Albumin	0.055+/-0.008*
Triolein	0.090+/-0.010*

Data are means +/- S.E.M. N=16-30 animals/group. The RES stimulants were administered according to the regimens in Methods. The plasmas were processed, and purified extracts of HDFx were administered to naïve animals, similar to the procedure in Table 6, i.e., 4 doses over 48 hr. Bloods were drawn for phagocytic indices on the 3rd day using 8 mg/100 g bw colloidal carbon.

*P<0.01 compared to controls.

Table 8. Effect of hemorrhage and trauma with and without HDFx pretreatment on cytokine levels in lymphocytes

Group	IL-2	IL-6	IFN-gamma
Controls-shams	402+/-75	1.7+/-0.3	7.5+/-0.3
Hemorrhage Alone	110+/-20*	0.35+/-0.12*	0.65+/- 0.11*
Hemorrhage+ HDFx	386+/-65	1.9+/-0.14	7.4+/- 0.4
Trauma Alone	75+/-15*	0.22+/-0.08*	0.21+/-0.05*
Trauma+ HDFx	382+/-76	1.6+/-0.4	7.3+/-0.4

Data are means +/- S.E.M. Values are given in U/ml. N=16-32 animals /group.

*P<0.001 compared to paired hemorrhaged, traumatized and control groups.

Table 9. Depletion of macrophages and NK cells but not PMNs decrease ability of plasma extracts from trauma-adapted animals to increase RES phagocytosis

Group	Plasma Extract on Phagocytic Index
Naïve Controls (saline)	0.028+/-0.002
Trauma-Adapted Controls	0.160+/-0.012*
Trauma-Adapted+Anti- Ly6G	0.148+/-0.010*
Trauma-Adapted+Anti-NK1.1	0.122+/-0.008**
Trauma-Adapted+ 2-chloroadenosine	0.050+/-0.006**

Data are means +/-S.E.M. Test dose colloidal carbon= 8 mg/100 g bw.

*P<0.001 compared to naïve saline controls.

**P<0.01(ANOVA) compared to all other groups.

in the Methods, PMN or NK cells were depleted, individually, by injection of anti-Ly6G or anti-NK1.1, respectively. Since, at the time of these studies, no specific antibodies were available for depleting macrophages, we utilized an i.p. injection of 2-chloroadenosine.

Table 9 serves to summarize these data. As can be seen, depletion of only the leukocytes failed, completely, to abrogate the manufacture of HDFx and, likewise, failed, completely, to prevent adaptation to traumatic shock. In contrast to this, depletion of the macrophages, resulted in an approximately 80 % loss of the manufacture of HDFx and curtailed dramatically the ability of rats to

adapt to the traumatic shock. Depletion of only the NK cells resulted in about a 15-20 % inhibition of the animals to generate HDFx and a concomitant small loss in adaptation to the trauma.

Effects of blockade of the RES-macrophage system on adaptation to trauma and levels of HDFx

RES- blocked animals, subjected to the trauma-adaptation procedure, clearly showed greatly attenuated plasma levels of HDFx (**Table 10**). Indeed, HDFx extracted from these rats was not able to boost phagocytic indices

Table 10. Blockade of the RES-macrophage system with diverse colloids decrease/inhibit ability of plasma extracts from trauma-adapted animals to increase phagocytosis and concomitantly abrogate adaptation

Group	Plasma Extract on Phagocytic Index	Abrogation(Yes/No)
Naïve Controls	0.045+/-0.005	--
Trauma-Adapted Controls	0.280+/-0.032*	No
Trauma-Adapted + Proferrin	0.055+/-0.006	Yes
Thorotrast	0.052+/-0.006	Yes

Data are means +/- S.E.M. Test dose of colloidal carbon =4 mg/100 g bw.

*P<.0.001 compared to all other groups (ANOVA).

when injected into naïve animals. Nor could the extracts increase the survival of animals subjected to traumatic shock or given endotoxin. Only 20-25 % of the former animals survived, while all the animals given the LD₅₀ dose of endotoxin died within 24-48 hr, a clear potentiation (i.e., 16 out of 16 rats died).

Discussion

Overall, we believe that, collectively, our experiments presented herein demonstrate the discovery of a new biological, immune-modulator and, potentially, powerful host defense molecule unlike any heretofore discovered agent. We believe it to be a conserved molecule, most likely, present in humans, as our preliminary experiments have demonstrated it to be present in mice, rabbits and guinea-pigs (unpublished data). Although we have not, as yet, been able to determine its complete molecular structure, it is a protein with a molecular weight between 35-40 KDa, it is non-dialyzable, heat stable at 60° C for at least 20 min, and larger than any known defined defensin peptides (which for the most part are less than 15 KDa in size) [for review, see 51] and much smaller than the large molecular weight molecules, such as fibronectin and complement C-molecules or their split products, all of which are important in host defense. Moreover, none of the known defense molecules can protect animals against all of the models of shock, trauma, or endotoxins used in our present study [51]. Although not shown, herein, repeated administration of purified HDFx to animals over a period of 6-months failed to result in any organ-tissue pathologies on autopsy or any pathophysiological change that were detectable (unpublished data).

Metchnikoff presented voluminous data to suggest that the body's major phagocytes (i.e., macrophages) protected animals against germs [52]. Up until his death, he believed the body contained a substance that played a central role in the relationship (and regulation) of macrophages to phagocytose germs and protect the organism from attack from numerous microbes [52]. Surprisingly, a long hiatus seemed to have taken place between Metchnikoff's early, fundamental works at the turn of the last century until George Mackaness' elegant work in the 1960s on macrophages [for review, see 53]. Our early work on RES-macrophages in the 1960s served as an important backdrop to the present discovery [25, 26, 32, 33], in which one of us established the fundamental role of this RES-macrophage system to host defense, and how its response could be quantified [25, 26, 28, 29, 37].

The present studies on the origin of HDFx seems to implicate fundamental roles for the RES-macrophage and NK cell systems in its synthesis for a number of reasons: 1) depletion of macrophages, but not leukocytes, results in a failure of the intact host to generate almost 80 % of its total concentration in response to profound lethal body stress; 2) depletion of the NK cells results in a loss of about 15-20 % of the host's ability to produce HDFx; and 3) blockade of the RES-macrophage system with diverse colloids abrogates most of its protection against lethal stresses and endo-toxins concomitant with a loss of biological activity. The fact that either depletion of the macrophages or depletion of NK cells, or blockade of the RES-macrophage system, results in a loss of animals to adapt to repeated lethal stress is, potentially, a very

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important finding which could have wide-sweeping implications in prevention of hospital-borne infections, new emerging diseases, parasitic diseases and food-borne diseases if a similar, conserved molecule is present in humans. In addition, since we have found HDFx in a number of mammals, it could prove to be quite useful in animal and pet care (and disease prevention). The ability of HDFx-stimulated animals to cope with lethal gram-negative endotoxins strengthens this hypothesis.

In view of our findings, we are tempted to speculate that HDFx could be quite useful against potential biotreats, amelioration of the effects of massive bodily injuries in both civilian and military personnel, and burn victims early after injury, where the chances for superimposed bacterial infections present great risks. Further, by virtue of HDFx's macrophage-stimulating abilities, it may prove useful in increasing the biological activities of infection-fighting cells in cancer patients who have had bone marrow transplants, and allow oncologists to decrease chemotherapy doses while increasing patient survival chances. In addition, due to HDFx's powerful immune-stimulating abilities, it could prove very useful as pretreatment for patients needing high-risk surgical procedures, particularly in the elderly.

For almost five decades, it has been known that various colloidal substances can, dramatically, stimulate the RES-macrophage system and enhance survival of diverse animals to different types of lethal stressors, bacteria, viruses, and endotoxins, among other agencies (for reviews, see 14, 15, 17, 18, 19, 30, 31, 54, 55]. However, until the present study no single substance has been demonstrated to be: 1) shown to be synthesized and released by these cells in response to diverse colloidal RES protective stimulants such as aggregate denatured albumin, triolein, and choline; 2) synthesized by these cells and concomitantly stimulate the RES-macrophages and induce protection against a diverse group of lethal stressors and gram-negative endotoxins; and 3) critical in adaptation to lethal traumatic shock. The synthesis and release of HDFx may, thus, be pivotal in allowing diverse RES colloidal stimulants to potently activate RES-macrophages and generate protection against diverse lethal stressors. Our new findings may also help to explain why some RES colloidal

stimulants are weaker than others. This diversity may simply be a reflection of a stimulant's ability to induce adequate cell concentrations of HDFx and, therefore, adequate blood levels of HDFx. Obviously, more testing will be required before this hypothesis can be substantiated.

Considerable evidence has accumulated to indicate that various physiological factors are important in allowing uptake and ingestion of particulate matter and microorganisms by the RES-macrophages [for reviews, see 14, 15, 17, 19, 24, 31]. Among these are opsonins, humoral-hormonal factors, vasoactive molecules, plasma factors such as fibronectins and complement products, as well as micro-circulatory-blood flow interactions. Whether or not HDFx stimulates or results in production/release of any of these physiological factors is entirely speculative at this time, but nevertheless should be considered as food-for-thought. Since HDFx only appears to demonstrate biological activity after an imposed severe bodily stress, and is released from the RES-macrophage and NK cell systems, studies should be undertaken to determine what mechanism(s) is involved in its release from these cells. Such information could prove useful in therapeutic applications.

Some discussion of our findings on lymphocyte cytokine storage and release, in response to the presence (and generation) of HDFx, appears to be in order here. Our studies clearly confirm the works of others who have demonstrated that shock-trauma result in a loss of various cytokines, such as IL-2, IL-6, and IFN-gamma, among others from lymphocytes [17, 19]. However, the present findings, unlike previous studies, demonstrates that the lymphocyte storage and release of these cytokines in states of shock-trauma appear to be related to the ability of the RES-macrophage system to generate HDFx. That is, in states of shock-trauma where levels of HDFx would be low or exhausted, there is clearly a massive release of IL-2, IL-6, and IFN-gamma, as shown by our present studies. However, when animals are pretreated with several injections of purified HDFx, there is no loss of these cytokines from the lymphocytes. It would appear from these findings that HDFx is a regulator of cytokine levels in lymphocytes. Cytokines have well-documented roles in diverse disease states, inflammatory conditions, circulatory shock, wound healing, burns,

sepsis, and trauma, among others [17, 19, 24, 56, 57, 58], which may be beneficial or detrimental depending on the type and amount of cytokine and its cellular origin. Our findings on the cytokines, lymphocytes, macrophages and HDFx may have some unique implications for the immune system. Activation of virgin helper T-cells produce /secrete a major cytokine, namely IL-2 [57, 58]. This production of IL-2 induces proliferation of more helper T-cells (a subtype, i.e., Th1 cells) and other cytokines, i.e., IFN-gamma and TNF-alpha [57, 58]. IFN-gamma is known to be capable of stimulating production and activation of macrophages as well as down-regulating (by negative feedback) another type of helper cell, i.e., Th2 cells which normally could then lead to production of other types of cytokines such as IL-4, IL-5, and IL-10 [57, 58]. Whether or not HDFx is a regulator of these pathways remains to be seen.

Since HDFx appears to be synthesized by RES-macrophages and the NK cells, but not by PMNs, we conclude that macrophages are the key effector cells for HDFx. To our knowledge, an innate host-defense regulator (such as HDFx) which can, collectively, counter gram-negative endotoxins, hemorrhage, and trauma by selective modulation of innate immunity without toxicities has not been reported previously.

This discussion could not be complete unless we attempted to address the implications of our findings for the NK cells. Normally, NK cells are not found in great supply unless a tissue is under attack [24, 57, 58]. Like the PMNs, NK cells are basically on-call. NK cells leave their storage sites (i.e., blood, liver, and spleen) and enter tissues at sites of infection. Once in infected tissues, they proliferate rapidly. In addition to being manufacturing sites for various cytokines, they can destroy virus-infected cells, tumor cells, bacteria, fungi, and parasites. Neither macrophages nor PMNs have all of these capabilities [57,58]. Our results demonstrate that NK cells are responsible for the generation of almost 20 % of HDFx production under the assault by various stressors and RES stimulants. At present, it is not clear what factor(s) regulate these important, somewhat mysterious cells of the innate immune system. It is tempting to speculate, in view of our findings, that HDFx may be one such regulator. Some preliminary experiments performed in our laboratories

may be useful here. We have found that rats and mice which spontaneously survive implantation, and exhibit reduction in tumor size, after introduction of sarcoma 180, exhibit high plasma levels of HDFx; whilst animals which are pretreated with purified HDFx exhibit increased survival (and reduction in tumor growth) after implantation of sarcoma-180 (unpublished data).

The latter findings may have direct implication for the growing number of reports in humans who spontaneously have survived cancers when all chemotherapeutic and radiation treatments failed to help these patients, and who were given what amounted to death notices [for review, see 59]. Whether these spontaneous remissions/survivors are due to stress-induced production of HDFx remains to be determined.

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