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NBT Reduction by Human Neutrophils Stimulated by Adenoviruses *in Vitro*[1] (38208)

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Previous investigators have demonstrated the reduction of nitro blue tetrazolium (NBT) dye by human neutrophils and have suggested the application of this phenomenon for the differentiation of bacterial infections (1, 2). In a recent study, some patients with acute respiratory disease (ARD) associated with laboratory evidence of adenovirus infections also demonstrated a positive NBT test (3). While superinfection with bacterial agents could not be completely ruled out, these data suggested that, in addition to bacteria, certain viruses may also be involved in the dye reduction effect. The following report relates our experience with NBT reduction by human neutrophils stimulated *in vitro* by adenovirus and tissue culture material.

Materials and Methods. White blood cells. Heparinized blood samples were obtained from several apparently healthy human donors. In some instances, multiple specimens were obtained from the same donor.

Virus preparation. Neutrophilic NBT reduction was stimulated by adenoviruses, types 4 and 7 (Ad-4, Ad-7). Both naturally occurring (wild) and attenuated vaccine strains of the two adenovirus serotypes were employed. Viruses were propagated in human, diploid, embryonic lung (WI-38) or in heteroploid epithelioid cells (HeLa). No adventitious agents could be detected in the former, but the latter cells are known to harbor mycoplasma.

Tissue culture maintenance media consisted of Eagle's minimum essential medium (4) reconstituted in Earle's balanced salt solution (EMEM). Initially, all EMEM was fortified with 2% fetal calf serum, but this was omitted for the trypsin digestion experiments.

Viruses were harvested when most of the cells exhibited adenoviral cytopathic effect. The cells were frozen and thawed three times, sonicated for 5 min in a Raytheon oscillator (9 kc/sec), and centrifuged at 1,000 rpm for 10 min. Supernatant fluids were aliquoted in 1 ml volumes and frozen as stock virus. Uninoculated tissue culture cells were prepared similarly for use as controls.

Assays for virus infectivity titers were carried out by a microtechnique (5). Fifty percent tissue culture (TCD_{50}) endpoints were calculated by methods of Kärber (6) and are expressed as $\log_{10}$ per ml.

Viral antisera. Type-specific antisera to strains of adenovirus, types 4 and 7, either wild or vaccine, were prepared in rabbits by the method of Van der Veen (7).

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Adenovirus infectivity neutralization (Nt) tests were carried out by a microtechnique (5).

NBT test. The NBT test employed the method of Matula and Paterson (2). Before use in the test, viruses or uninoculated tissue culture materials were filtered through a 0.2 μ Millipore filter. *Escherichia coli* endotoxin[2] (200 μg), a known neutrophil stimulator, was used as a positive control.

Five-hundredths (.05) ml of endotoxin, virus, or normal tissue culture material was mixed with an equal volume of 0.2% NBT[3] saline solution in a siliconized concave microscope slide. One-tenth (0.1) ml of heparinized donor blood was then added to the virus-NBT mixture and after thoroughly mixing with a capillary tube incubated at 37° for 25 min. At the end of the incubation period, the test was again thoroughly mixed. Duplicate smears of the mixture were examined under high power magnification (1,000×) for dense or stippled cytoplasmic inclusions of reduced dye (formazan). The NBT response was expressed as percent neutrophils containing formazan deposits.

Results. Response of donor neutrophils to various stimuli. Table I compares the neutrophilic NBT reduction responses to the dye alone, to *E. coli* endotoxin, and to uninoculated cell culture material. Differences in magnitude of responses were observed, not only among donors, but also among the sequential blood specimens obtained from an individual donor. Background counts of "NBT-alone" ranged from 2% to 23%, but the higher values were not always associated with an elevated endotoxin or tissue culture response. Generally, the responses to endotoxin stimulation exceeded response to the "NBT-alone" by a factor greater than 2-fold; thus, they were taken as valid positive controls for neutrophilic NBT-reduction.

In most instances, the tissue culture controls tended to be higher than the "NBT-alone" values, especially with HeLa cells maintained in serum-free media. All cell controls, however, were much lower than those of the endotoxin controls, and were, therefore, considered to be acceptable for negative controls. Correcting the values of the positive (endotoxin) and negative (cell culture) controls by substracting the "NBT-alone" value resulted in a narrower range distribution and smaller standard deviation for the positive and negative responses. Therefore, in subsequent analysis of data, this correction was applied. Furthermore, in view of the great variance of responses, each test was interpreted by comparing the various responses of test virus material with the values for positive and negative controls obtained in the concomitant experiment.

Demonstration of neutrophil stimulation by wild adenoviruses. Table II shows the results of tests with wild and vaccine strains of adenoviruses, types 4 and 7, on NBT-reduction by neutrophils obtained from various donors. All tests indicated increased NBT-reduction when neutrophils were mixed with virus material derived from wild stock viruses, but not with the homologous vaccine strains. In several tests involving stimulation by wild viruses, the reduction of NBT by neutrophils was greater than that produced by endotoxin. All tests for wild virus stimulation showed responses which were 2–23-fold (mean = 6.6×) greater than the values produced by the negative control tissue cultures (WI-38). This was compared to the 2–39-fold (mean = 9.1) difference between endotoxin stimulation and the negative controls. These data (Table II) also indicate that the enhanced response to wild Ad-7 compared to WI-38 cell controls was greater (mean = 10.2×) than to wild Ad-4 (mean = 3.0×). This difference may be due to the lower cell control values found in the former tests. No enhancement of NBT-reduction was noted in response to the vaccine virus strains, and in some instances, their positive cell count was lower than that obtained with normal WI-38 cells.

Tests of these adenovirus strains propagated in HeLa cells showed essentially the same relative difference in the ability of

[2] *E. coli* endotoxin 0.26.B6, Difco, Detroit, Michigan.

[3] NBT 484235, Calbiochem, LaJolla, California.

TABLE I. NBT-Reduction Responses by Human Neutrophils to Various Stimuli *in Vitro*.

		Percent neutrophils with reduced NBT corrected values[a]				
				Uninoculated tissue cultures		
WBC donor	Sample number	NBT alone	NBT + *E. coli* endotoxin	WI serum[b] media	HeLa serum[b] media	HeLa no serum media
JM	1	5	25	14	ND[c]	ND
RP	1	12	34	2	ND	ND
PK	1	8	35	14	ND	ND
PK	2	9	21	3	ND	ND
GD	1	6	23	13	ND	ND
GD	2	10	22	5	2	ND
GMC C	1	20	22	5	ND	ND
GMC C	2	15	28	5	ND	13
GMC C	3	4	49	ND	ND	24
EE	1	4	43	3	ND	ND
EE	2	2	39	ND	ND	25
EE	3	10	49	ND	ND	20
MR	1	2	19	9	ND	ND
MR	2	8	36	12	3	ND
MR	3	6	23	6	ND	ND
MR	4	9	51	ND	ND	11
PM	1	16	40	6	ND	ND
PM	2	15	43	7	7	ND
PM	3	20	39	0	ND	ND
PM	4	5	57	ND	17	33
PM	5	9	49	ND	ND	21
PM	6	23	43	ND	ND	0
		Corrected values[a]				
Range		(2–23)	(19–57)	(0–14)	(2–17)	(0–33)
Mean		9.91	36.41	6.93	7.25	18.38
Standard error		1.27	2.37	1.16	3.43	3.83

[a] Corrected values (% positive neutrophils less "NBT alone" value).
[b] EMEM maintenance media with 2% fetal calf sera.
[c] ND = not done.

wild, but not vaccine strains to enhance reduction of NBT by the neutrophils.

Since infectivity assays of the wild and vaccine virus had indicated that the Ad-4 vaccine strain had less virus (Table II), the homologous type wild strain was diluted 10-fold to present more comparable infectivity titers *vis-à-vis* the vaccine strain. This dilution produced only a slight reduction in the percent of neutrophils showing NBT-reduction (46% vs 40%).

Effect of dilution on stimulation effect. Aliquots of virus material processed as described above (with an infectivity titer of $10^{4.1}$ ml) were diluted with uninoculated tissue culture fluids (HeLa or WI-38). A 10-fold dilution of any of the wild adenovirus produced only a slight decrease in percent of neutrophils demonstrating NBT-reduction. When diluted 30-fold or greater, the enhanced stimulatory effect of the virus over the normal cell control value was eliminated.

Heat-lability of adenovirus stimulatory

TABLE II. Percent of Neutrophils with Reduced NBT after Addition of Various Substances.

	White blood cell donor												
					PK Exp		MR Exp		PM Exp		GM Exp		
	JM	GD	EE	RP	1	2	1	2	1	2	1	2	Mean
Controls													
NBT only	5	6	4	12	9	9	2	6	16	20	20	15	10
	Corrected values[a]												
NBT + *Escherichia coli* endotoxin	25	23	43	34	35	21	19	23	40	39	33	28	30
NBT + WI cells	14	12	3	2	13	3	10	6	6	−2	5	5	7
Tests													
Wild Ad-4[b]	33	30	ND[c]	ND	32	ND	22	ND	27	ND	18	ND	27
Vaccine Ad-4[d]	8	7	ND	ND	10	ND	6	ND	6	ND	4	ND	7
Wild Ad-7[d]	ND	ND	27	29	ND	16	ND	23	ND	23	ND	26	24
Vaccine Ad-7[d]	ND	ND	6	4	ND	2	ND	8	ND	1	ND	7	5

[a] Corrected values (percent positive neutrophils less NBT control values).
[b] Infectivity titer = 4.1/ml.
[c] ND = not done.
[d] Infectivity titer = 3.1/ml.

TABLE III. Effect of Heat on Adenovirus Stimulation of Neutrophilic NBT-Reduction.

	White blood cell donor						
	EE	GM	PM	PK	RP	MR	Mean
Controls							
NBT only	4	15	20	9	12	6	11
			Corrected values[a]				
NBT + *Escherichia coli* endotoxin	43[a]	28	39	21	34	23	31
Cell culture	3	5	−2	3	2	6	3
Tests							
Unheated							
Wild Ad-7	27	16	23	16	29	23	24
Vaccine Ad-7	6	7	1	2	4	8	5
Heated[b]							
Wild Ad-7	4	4	−1	2	5	8	4
Vaccine Ad-7	3	3	0	3	2	10	4

[a] Corrected values (percent positive neutrophils less NBT control values).
[b] 56° for 60 min.

effect. Table III shows the effect of heating adenoviruses at 56° for 60 min on their capacity to stimulate neutrophil reduction of NBT. No infectious virus could be detected after heat treatment. The data show that heating reduced the percentage of neutrophils responding to adenovirus stimulation to a level similar to that produced by uninoculated cell controls. Heat treatment had little or no effect on the response of vaccine virus strains or to uninoculated cell controls, nor did it diminish the stimulatory effect of the *E. coli* endotoxin.

Trypsin-lability of adenovirus stimulatory effect. Table IV shows the results of trypsin treatments on adenovirus material and uninoculated cellular debris. Viruses, endotoxin, and uninoculated cell cultures were prepared and trypsinized with crystalline enzyme (1 mg/ml specimen) by the method described by Rowe (8). The data demonstrate that the exaggerated stimulatory effect of wild adenovirus (either type 4 or 7), was abolished by the trypsin treatment. No reduction of activity was noted with vaccine viruses or uninoculated cell controls either after similar treatment with trypsin, or after sham enzymatic treatments (same process except trypsin omitted). Titers of infectious adenovirus before and after trypsin or sham treatments varied. Wild or vaccine Ad-4 infectivity titers were reduced 90% and 99%, respectively, by the digestion process (with or without enzyme), whereas, no reductions in either wild or vaccine Ad-7 titers were observed.

Serum neutralization of the stimulatory factor. Table V shows the effect of treatment with rabbit antisera to wild and vaccine adenovirus strains on the stimulatory factor associated with wild adenoviruses. Antisera was diluted to contain 20 units per ml of infectivity neutralizing antibody to the homologous virus. Normal rabbit sera (diluted 1:10) was included as a control.

Equal volumes of wild type 4 or 7 adenovirus fluids were mixed with their respective sera and incubated for 1 hr at 37°. Aliquots of the mixture were then tested for stimulatory activity (neutrophilic NBT-reduction) as previously described.

It was observed (Table V) that the viruses (both type 4 or type 7) lost their capacity to enhance neutrophilic NBT-reduction when treated with either of the antisera to the wild adenovirus. Antisera prepared against the vaccine strains, however, had no effect on stimulation of NBT-reduction. The neutralization effect appeared to be cross-reactive between the 2

TABLE IV. Effect of Trypsin Treatment on Adenovirus NBT-Reduction Factor and Infectivity Titers.

	Controls					Tests											
	Corrected values[a]					NBT stimulation-corrected values[a]											
			NBT + uninoculated HeLa cell[b]			Wild Ad-4			Vaccine Ad-4			Wild Ad-7			Vaccine Ad-7		
WBC donor	NBT only	NBT + *Escherichia coli* endotoxin	Untreated (U)	Trypsinized (T)	Sham (S)	U	T	S	U	T	S	U	T	S	U	T	S
EE	2	40[b]	25	27	26	42	18	37	24	26	20	36	21	34	23	22	20
GM	4	49	24	22	21	45	23	34	23	22	19	35	19	30	24	25	20
						Infectivity titer/ml											
						4.1	2.4	2.1	3.1	2.1	2.1	3.1	3.1	3.1	3.1	3.1	3.1

[a] Corrected values (percent positive neutrophils less NBT control values).

[b] No calf sera in maintenance media.

wild types (4 and 7). Subsequent titration of the neutralizing potency of the two antisera to the wild type adenoviruses showed that a 50% reduction in stimulatory activity could be demonstrated at dilutions as high as 1:50–1:80 for either homologous or heterologous wild type adenoviruses. This crossing, however, was more pronounced with the type 4 antisera; i.e., type 4 antiserum neutralized type 7 virus to a higher degree than did type 7 antiserum used against type 4 virus.

No heterologous crossing in infectivity neutralizing antibody could be demonstrated with these antisera. On the other hand, antisera to the homologous type, either vaccine or wild, completely neutralized the infectivity of either wild or vaccine virus of the homologous type.

No enhanced stimulatory activity could be demonstrated with the vaccine viruses, nor was there any reduction in the low activity of these strains after mixing with any of the antisera to either wild or vaccine adenoviruses.

Discussion. The neutrophil NBT-reduction test, when performed on patients' WBCs, has been proposed as a method for the differentiation of bacterial infections (1, 2). Some investigators have indicated that patients with viral diseases may also show increased neutrophil NBT-reduction, but to a lesser extent than persons with untreated bacterial infections (9). In our experience, about 30% of a group of Navy recruits with complaints of ARD associated with laboratory confirmed adenovirus infections demonstrated spontaneous neutrophilic capacity to reduce the NBT-dye (3). Recent investigations have also demonstrated that patients with mycoplasma infections may also exhibit this phenomenon (10). The purpose of the present investigation was not to determine the specificity of the NBT test, but rather to determine whether the NBT-reduction phenomenon could be produced using viruses as *in vitro* stimulators in a manner similar to that reported by Matula and Paterson with *E. coli* endotoxin (2). Under the conditions described in this report, we have clearly demonstrated that the reduction phenomenon could be induced by

TABLE V. Serum Neutralization of Adenovirus NBT-Reduction Stimulatory Factor in Human Neutrophils.

	Percent stimulative activity remaining after serum and virus mixture[a]				
Stimulator	Normal	Antiwild Ad-4	Antivaccine Ad-4	Antiwild Ad-7	Antivaccine Ad-7
Wild Ad-4	100	0	92	25	100
Wild Ad-7	100	0	100	33	100

[a] Twenty infectivity neutralizing units of respective hyperimmune sera or 1:10 dilution of normal rabbit serum.

adenoviruses *in vitro,* and that the responsible substance was probably a protein associated with wild virus strains, but not with vaccine viruses.

The stimulatory activity was abolished by heat or trypsin, was nondialyzable, and was neutralized by antisera prepared against wild strains of either type 4 or type 7. No neutralization was observed with antisera to vaccine strains (4 or 7), although these sera completely neutralized the infectivity of either wild or vaccine strains of the homologous type.

The exact nature of the stimulatory factor is of great interest. Apparently it is a protein component of the adenovirus capsid or a substance associated with viral modified host cell products. It does not appear to be associated with a particular cell culture type, since no difference between the WI-38 or HeLa cell substrates was observed. The possibility that other agents indigenous to the host cell, i.e., cell wall defective bacteria or mycoplasma may be the responsible factor is also precluded since vaccine virus propagated in these cell cultures did not exhibit the stimulatory effect. Whether continued passage of vaccine virus in these cells would eventually result in enhanced stimulation of NBT-reduction remains to be determined. It is conceivable, however, that bacterial and mycoplasm organisms, which sometimes contaminate cell cultures, may contribute to the high NBT values produced by uninoculated cells. In any event, the stimulatory phenomenon we have observed appears to be virus-directed, but independent of the viral infectious particle.

Previous investigators have described the early cytopathic effect (ECPE) of adenoviruses when cultured in cells (8, 12, 13). This effect has been ascribed to a protein toxin associated with the vertex capsomeres (penton bases) of the adenovirion (13, 14). In some respects (i.e., trypsin and heat-lability) the stimulatory factor resembled this toxic component. In addition, the cross-reactivity of the two heterotypic wild type virus antisera in toxin neutralization is consistent with the group specificity of the antigen associated with the penton bases (13). It would be of interest to determine more precisely the relationship of the penton bases of the virion to the NBT-reduction stimulatory factor.

We postulate that the stimulatory factor is a biologically active virulence marker of certain naturally occurring adenoviruses. Passage of these strains in some cell cultures may modify production of the toxic component and result in attenuation of the virus.

The significance of the adenoviral toxin in human disease has been little investigated since it was reported as a tissue culture cytopathic effect 15 years ago. By use of the NBT test, it may be now possible to determine whether the presence or absence of the toxin is related to the severity of symptoms associated with adenovirus acute respiratory disease.

A practical use of the adenovirus NBT test may be in differentiating wild from attenuated vaccine adenoviruses. To date, no means of separating these strains, either biophysically or serologically, was available.

Whether separation is now possible or is peculiar to the strains employed in this study is now under investigation.

Summary. Adenoviruses were shown to stimulate the reduction of NBT-dye by human neutrophils *in vitro*. This factor was associated with naturally occurring, but not attenuated vaccine strains of adenovirus, types 4 and 7. The stimulatory factor was inactivated by heat and trypsin and neutralized by homotypic and heterotypic hyperimmune rabbit antisera against wild virus, but not by antisera to vaccine viruses. Some of the characteristics of the stimulatory factor are similar to those previously described for adenoviral toxins associated with penton bases of the virion. The implications of these phenomena in the differentiation of vaccine from wild type viruses and in pathogenesis of adenovirus disease are discussed.

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