DNA Binding to Human Leukocytes

Evidence for a Receptor-mediated Association, Internalization, and Degradation of DNA

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Abstract

Previous studies have indicated that white blood cells possess DNA on their outer membranes. In this study we set out to determine whether exogenous DNA bound to cells in a fashion compatible with a ligand receptor union. Purified populations of white blood cells; neutrophils (polymorphonuclear leukocytes, PMN), adherent mononuclear cells (ADMC), rosetting lymphocytes (E+ cells), and nonrosetting lymphocytes (E− cells) were incubated with radiolabeled lambda phage DNA in increasing concentrations. Binding of [^{3}H]DNA was a saturable process and was inhibited by excess cold DNA and prior trypsinization of the cells. Rate zonal density centrifugation of purified cell membrane preparations confirmed that DNA was binding to the outer cell surface. The dissociation constant for all four cell types was $\sim 10^{-9}$ M, and from 0.81×10^3 to 2.6×10^3 molecules of lambda phage DNA bound to each cell depending upon cell type. Binding was *not* competitively inhibited by RNA, polydeoxyadenylic acid-polydeoxythymidylic acid (poly [d(A) · d(T)]), or mononucleotides. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE)-separated proteins from PMN, ADMC, E+, and E− cells were electrophoretically blotted onto nitrocellulose sheets; a probe of biotin-labeled DNA indicated a single species of DNA-binding molecule migrating in a position consistent with a molecular weight of 30,000. Isotopic and immunofluorescent studies indicate that DNA is internalized and degraded to oligonucleotides; this process is inhibited by cycloheximide. These results support the notion that there is a common binding site for DNA on white blood cells, that the stoichiometry of the association is compatible with a ligand receptor relationship, and that this apparent receptor is responsible for the endocytosis and degradation of exogenous DNA.

Introduction

Increasing evidence shows that peripheral blood cells can both excrete DNA (1–6) and have DNA associated with their plasma membrane fraction (7–11). DNA receptors have been described on human platelets (12) and would appear to have a functional role, as their association with DNA leads to platelet aggregation and serotonin release (13). Whether the interaction of DNA with the membranes of nucleated peripheral blood cells is of functional importance has not been well studied: Russell et al. (14) have presented evidence indicating that the suppression of leukemia in AKR mice depends on the presence of cell membrane DNA (cmDNA),[1] and Jachertz et al. (15) have performed experiments that would seem to show that DNA, released by antigen stimulated lymphocytes, can be transcribed into an RNA that encodes for a specific antigen binding protein. On the other hand, this general area of research may be only an epiphenomenon resulting from the release of DNA by dying cells (16) and its nonspecific adherence to cell membranes. Our group has presented evidence that the cmDNA is a probable candidate for a lactoferrin receptor (17), and that the interaction of lactoferrin with monocytes (18) functions in inhibiting the release of a monokine that stimulates T cells to produce granulocyte-macrophage colony stimulating factor (19, 20). In the present study we provide further evidence that cmDNA is not a nonspecific epiphenomenon by showing that (*a*) the interaction of DNA with cell membranes is compatible with a ligand receptor relationship, and (*b*) the apparent receptor is a protein of 30,000 mol wt, and (*c*) exogenous DNA is internalized and degraded under conditions that implicate metabolically active receptor regeneration.

Methods

Agents used. Lambda phage DNA and DNA polymerase 1 were purchased from Bethesda Research Laboratories (Gaithersburg, MD). DNA was dissolved in 10 mM Tris HCl, pH 7.5, 120 mM NaCl, and 0.1 mM EDTA. Just before use, the solution was heated to 60°C for 10 min and then quickly cooled on ice, to restore the DNA to its linear form. (In storage it tends to form circles due to "sticky" ends.) dNTPs were purchased from Sigma Chemical Company (St. Louis, MO); [^{3}H]dTTP (62 mCi/mg) was purchased from Amersham Corp. (Arlington Heights, IL); and DNase 1 was purchased from Worthington Diagnostics Div., Millipore Corp. (Freehold, NJ). Bovine serum albumin (BSA), transfer RNA, trypsin, Hepes, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol, sodium dodecyl sulfate (SDS), Tween 20, polyvinyl pyrollidine, and Triton X were purchased from Sigma Chemical Co. Hanks balanced salt solution (HBSS) was purchased from Gibco (Grand Island, NY). Ficoll-Paque was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Molecular weight standards for electrophoresis, polyacrylamide, N_1N^1-methylene-bisacrylamide, tetramethylenedianine, and ammonium persulfate were purchased from Bio-Rad Laboratories (Richmond, CA). Biotinylated DNA (B-DNA) was prepared from lambda phage DNA using nick translation to incorporate biotinylated *d*-uridine triphosphate (UTP) (21); the reagents for this procedure (d-UTP, streptavidin-biotinylated horseradish peroxidase complex, DNA polymerase, and DNase 1) were purchased in kit form from Enzo-Biochem, Inc. (New York, NY) and processed as per the supplier's instructions. Goat antibiotin

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1. *Abbreviations used in this paper:* ADMC, adherent mononuclear cells; B-DNA, biotinylated DNA; cmDNA, cell membrane DNA; cmS, cell membrane suspensions; dsDNA, double-stranded DNA; E+ cells, rosetting cells; E− cells, nonrosetting cells; NCS, nitrocellulose sheets; NHNP, nonhistone nuclear proteins; PAGE, polyacrylamide gel electrophoresis; PBMC, peripheral blood mononuclear cells; PMN, polymorphonuclear leukocytes; PMSF, phenylmethylsulfonyl fluoride; RBC, erythrocytes; SSC, standard saline citrate buffer; TCA, trichloroacetic acid.

and rabbit fluoresceinated anti-goat were purchased in kit form from Enzo-Biochem, Inc.

Radiolabeling of DNA. DNA was radiolabeled by nick translation (22). In brief, the reaction was performed on 12.5 μg of DNA in a final volume of 250 μl. The incubation buffer was 50 mM Tris HCl, pH 7.9, 10 mM $MgCl_2$, 10 mM dithiothreitol, 50 μg/ml BSA, and all four deoxynucleoside 5'-triphosphates. The concentration of unlabeled triphosphates (dATP, dGTP, dCTP) was 300 μM; the concentration of [^{3}H]deoxy-thymidine triphosphate was 1.33 mM. The reaction was initiated by adding *Esterichia Coli* DNA polymerase (5 U) and DNAase I (100 pg); incubation was for 30 min at 16°C. The reaction was terminated by adding EDTA (final concentration, 20 mM). After we added carrier DNA and sodium acetate (0.1 M, final concentration), the DNA was precipitated by the addition of three times volume of ice-cold absolute ethanol (−70°C, 10 min). After being washed two times in absolute ethanol, the dried pellet was gently dissolved in 1 ml HBSS; this process took ~48 h. The procedure for labeling with ^{125}I is identical, but uses ^{125}I-dCTP and a 60-min incubation at 16°C.

The integrity of the labeled DNA was checked by (*a*) electrophoresis in a 1% agarose gel (buffer: 40 mM Tris-HCl, 20 mM Na acetate, 2 mM EDTA, 18 mM NaCl, pH 8.0; current, 40 mA; time, 6 h) using unlabeled DNA and a Hind III digest of lambda phage DNA as a reference, and (*b*) rate zonal density sedimentation of labeled and unlabeled lambda phage DNA: 5–35% sucrose in 1 M NaCl, 0.001 M EDTA, 0.02 M Tris-HCl, pH 7.5 buffer; SW 50.1 rotor − r max, 10.73 cm; r min, 5.97 cm; 28,000 rpm, 17 h.

We used the [^{3}H]DNA as a tracer in the binding studies, by mixing it in a constant proportion to cold lambda phage DNA to give ~8×10^4 cpm/μg of total DNA.

Preparation of peripheral blood cell populations. Human buffy coats (45 g) were obtained from the local Red Cross within 2 h of collection and were separated into adherent mononuclear cells (ADMC), rosetting cells (E+ cells), nonrosetting cells (E− cells), neutrophils (polymorphonuclear leukocytes; PMN), and erythrocytes (RBC) using standard techniques (17, 18). All cell preparations were assessed for viability, both before and at the end of all experiments, by Trypan Blue exclusion. Viability was >97% in all experiments. E+ cells were 98% pure as assessed by OKT3 (Ortho Pharmaceutical, Raritan, NJ). E− cells were 95% pure as assessed by staining for surface immunoglobulins with F(ab)$_2$ anti-human Fc (Kallestad Laboratories, Inc., Austin, TX), and ADMC were 95% pure as assessed by alpha-naphthyl esterase staining (Sigma Chemical Co.).

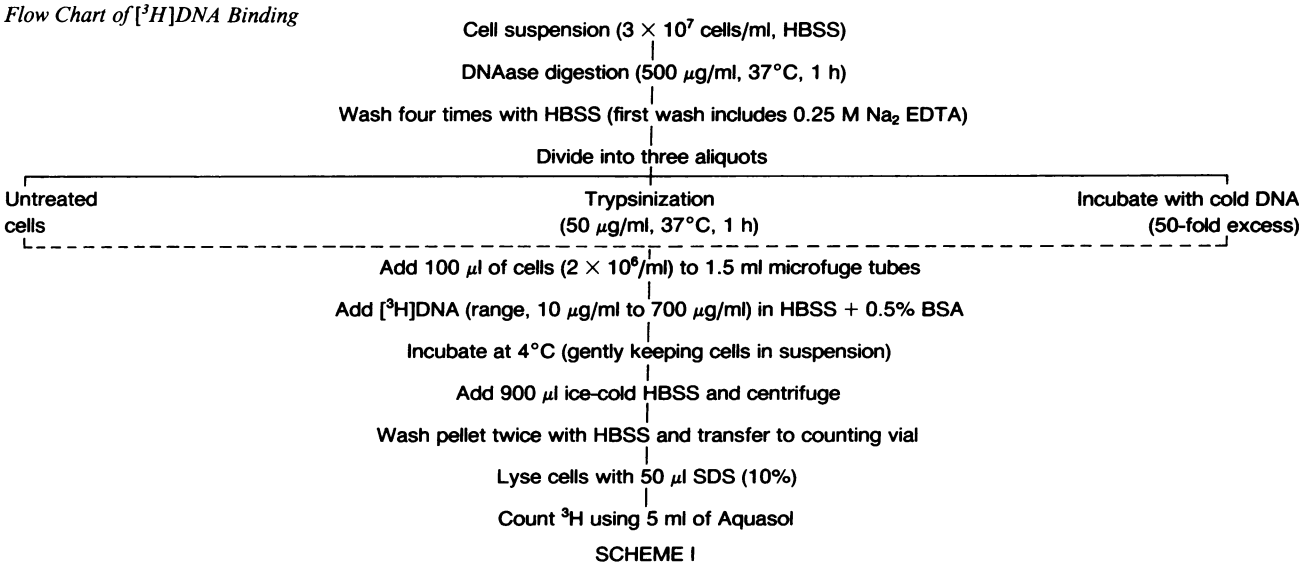
[^{3}H]DNA binding to cells. All binding studies were performed at 4°C according to the protocol outlined in Scheme I. Each experiment contained two controls, namely, trypsinized cells and a 50-fold excess of cold lambda phage DNA (this was added after boiling for 10 min to produce single-strand DNA; at the concentrations used, double-strand DNA [dsDNA] was too viscous). Bound DNA was calculated from the percentage of counts bound after subtracting the counts of nonspecific binding. The input DNA was plotted against the bound DNA using the CURFIT program (Hewlett-Packard Co., Palo Alto, CA); experiments with a closeness of fit (R^2) less than 0.85 were rejected. From the idealized curve generated by CURFIT, a standard Scatchard plot analysis (23) was used to estimate the binding affinity (K_d) and the number of molecules bound.

To further characterize the binding of DNA to cell surfaces, we modified the basic binding assay: the cells were subjected to enzymatic treatment as previously described (17), cells were preincubated with ligands that might exhibit a competitive inhibition, and cells were pulsed with cold DNA to determine if this modulated ligand binding.

Preparation of cell membranes. Cell membranes used in binding studies and receptor characterization were prepared according to the method of Maeda et al. (24). Briefly, 10^8 DNase-treated cells were resuspended in a 3:1 vol/vol of homogenization buffer—10 mM phosphate (pH 7.4), 1 mM dithiothreitol, 0.005 M PMSF, 10 mM sodium azide, 10 μg/ml DNase 1, and 1 mM $MgCl_2$ and 30 mM NaCl. The cells (kept on ice) were disrupted by 2- to 5-s bursts at power setting #7 of a Polytron homogenizer (Brinkmann Instruments Co., Westbury, NY). Cell disruption was monitored by phase contrast light microscopy to a level of 90% disruption. The homogenate was then layered over 4% sucrose made up in the homogenization buffer and centrifuged at 95,000 *g* for 1 h in a swinging bucket rotor (model SW 27; Beckman Instruments, Inc., Fullerton, CA). The isolated cell membranes collected at the buffer/sucrose interface as a readily identifiable white band. The membrane layer was harvested and diluted with a threefold excess of homogenization buffer and washed twice in the same buffer before storage in 35% sucrose at 4°C. This method yields membranes enhanced for 5'-nucleotidase (1:16); adenylate cyclase (1:22) and Na+-ATPase (1:15), compared with the initial homogenate. NADPH-cytochrome reductase and succinate dehydrogenase were not detectable.

Isolation of cmDNA. cmDNA was isolated from a purified preparation of cell membranes from 10^8 neutrophils as follows. The cell membranes were disrupted by a 1% solution of deoxycholate (37°C, 1 h). The solution was cleared by centrifugation and the supernatant was dialyzed against HBSS. The dialysate was treated with RNAase (heated to 80°C, for 10 min, to inactivate any contaminating DNAase), 10 mg/ml, 37°C, 3 h. Pronase (1 mg/ml) was then added and the mixture was incubated overnight at 37°C. Small oligonucleotides were separated by the Hirt extraction technique, by adding SDS to a final concentration of 1.5% and raising the molarity to 1 M NaCl. The resulting slurry was collected by

Flow Chart of [^{3}H]DNA Binding



SCHEME I

gentle centrifugation and extracted three times with chloroform/phenol (1:1) followed by further extraction three times with chloroform/isoamyl alcohol (24:1). DNA was precipitated by adding 3 vol of ice-cold absolute ethanol (−70°C, 10 min). One portion of this DNA was labeled with ^{3}H by the method of nick translation (22), and another portion was subjected to rate zonal density gradient sedimentation (5–35% sucrose, SW 50.1 rotor, 28,000 rpm, 17 h, 4°C) employing internal molecular weight markers of lambda phage DNA and a Hind III digest of lambda DNA. The sucrose solutions were made up in 1 M NaCl, 0.001 M EDTA, 0.02 M Tris HCl, pH 7.5. The [^{3}H]cmDNA was used in a dose-response binding study, using neutrophils as described above.

Preparation of nonhistone proteins and cytosol. We prepared chromatin from peripheral blood mononuclear cells (PBMC) (10^7 in HBSS) by disrupting the cells with a Polytron homogenizer (cell suspension on ice, power setting #8, 45 s). The homogenate was suspended in 10 ml of 66% sucrose and layered on 30 ml of 45% sucrose. The nuclei were pelleted by centrifugation at 800 rpm, for 30 min at 4°C, and the cytosol was saved. The nuclear pellet was washed once in PBS containing 0.25 M EDTA and the nuclei were hypotonically lysed by washing four times in 0.01 M Tris-HCl, pH 8.0. Chromatin was pelleted by centrifugation through 45% sucrose (50,000 *g*, 2.5 h, 4°C) and homogenized in 1 ml of 0.1 M EDTA and 1 ml of 2 mM Tris-HCl, pH 7.5. After "dehistonization" by extraction with 0.4 N sulfuric acid (15 min, 4°C), the pellet was rehomogenized in 2 mM Tris-HCl buffer containing 0.1 M Mg^{++} and Ca^{++} and treated with DNAase (25 μg DNAase 1, 37°C, 60 min). Proteins were precipitated with 0.4 N perchloric acid (10 min, 4°C) and pelleted by centrifugation. The resulting pellet was dissolved in 2 ml of 2% SDS before polyacrylamide gel electrophoresis (PAGE). The cytosol fraction was also dissolved in 2% SDS before PAGE.

Cell membrane binding studies. Cell membrane suspensions (cmS) were stored at 4°C in 35% sucrose until used. The sucrose was removed by washing three times in HBSS, and the cmS was adjusted to a total protein concentration of 250 μg/ml. The cmS was incubated with [^{3}H]DNA (37°C, 30 min) and washed twice with 30% sucrose solution. In control experiments the membranes were: (*a*) treated with trypsin (500 μg/ml, 37°C, 45 min), and (*b*) disrupted with deoxycholate (1% wt/vol, 37°C, 1 h). The treated membranes were subject to rate zonal density sedimentation (10–45% sucrose, 100,000 *g*, 3 h, 4°C). All sucrose gradients were fractionated from the top downward using a Buchler Densi-Flow II (Searle Analytic Inc., Fort Lee, NJ).

Detection of DNA binding proteins. A search for a putative DNA receptor was initiated using a modification of the method of Bowen et al. (25) to detect DNA binding proteins as we have previously described (26). Cell membranes, nonhistone nuclear proteins (NHNP), and cytosol were boiled for 2 min in a 0.06-M Tris-HCl buffer (pH 6.8) containing 2% SDS. The samples were applied (with 20% glycerol and a 0.001% bromophenol blue tracking dye) to a polyacrylamide slab gel (9% polyacrylamide in 3.75 M Tris-HCl buffer, 0.1% SDS, pH 8.8; stacking gel, 3% polyacrylamide in 0.125 M Tris HCl buffer, 0.1% SDS, pH 6.8) at a protein concentration of 50 μg per well for cell membranes, 150 μg for cytosol, and 25 μg for NHNP. The gels were run in a cold room at a constant current of 40 mA for ~2 h. The separated cell membrane proteins were electrophoretically transferred to nitrocellulose sheets (NCS) using a Trans Blot apparatus (Bio-Rad Laboratories). NCS were prewetted with transfer buffer (25 mM Tris HCl, 192 mM glycine, 20% methanol, pH 8.3) and apposed precisely to the polyacrylamide gels. Gels and apposed NCS were electrophoresed in transfer buffer at a constant voltage of 58 V for 1 h. Detection of DNA binding proteins was performed as follows: NCS with transferred proteins were immersed (30 min, 25°C) in standard binding buffer (10 mM Tris HCl, pH 7.0, 0.02% BSA, 0.02% Ficoll, 0.02% polyvinyl pyrollidine, 1 mM EDTA, 0.05 M NaCl, and 1% Triton X). After being washed twice in the same buffer, B-DNA was added at a concentration of 1.0 μg/ml in sufficient volume to just cover the NCS. After incubation at 25°C for 1 h, the NCS was washed twice in standard binding buffer and incubated with PBS, 2% BSA, 0.1% Triton X at 37°C for 30 min. Thereafter, streptavidin-biotinylated horseradish peroxidase complex was incubated (37°C, 30 min) with the NCS (0.02 ml/cm^2). After washing three times (5 min each wash) in 0.5 M NaCl, 10 mM phosphate buffer, pH 6.5, 0.1% BSA, Tween 20 (0.5 ml/liter); the NCS were further washed twice in double strength standard saline citrate buffer (SSC), 0.1% BSA, Tween 20 (0.5 ml/liter). The NCS were developed with fresh DAB reagent (5 mg diaminobenzidine tetrahydrochloride in 10 ml of 10 mM Tris-HCl buffer, pH 7.5, plus 200 μl of 1% cobalt chloride); then they were left in the dark for 10 min at 0°C before we added 15 μl of 30% hydrogen peroxide solution. This developer was applied to NCS (0.02 ml/cm^2) and kept in the dark until DNA binding proteins appeared as blue bands (this required ~10 min). Control experiments were (*a*) omission of B-DNA step, (*b*) cell membranes treated with trypsin, (*c*) 20-fold excess of nonbiotinylated DNA, and (*d*) erythrocyte membranes. Standard protein markers (phosphorylase, BSA, ovalbumin, and carbonic anhydrase) were run at the same time and electrophoretically blotted. This section of the NCS was cut out separately and stained with a protein silver stain (Bio-Rad Laboratories).

Functional studies. To determine whether membrane-bound DNA was internalized, we performed experiments using both radiolabeled DNA and biotin-labeled DNA with subsequent immunofluorescence.

In the isotopic experiments we stripped ADMC of their cmDNA (DNAase, 500 μg/ml, 37°C, 1 h) and after washing them three times we resuspended them in HBSS at a concentration of 10^7/ml. [^{3}H]DNA was added at a saturating concentration of 700 μg/ml and the cells were incubated at 37°C in a water bath. At timed intervals a 100-μl aliquot of cell suspension (10^6 cells) was removed and the cells were washed three times with a 0.2-M glycine buffer, pH 2.8, containing 200 μg of heparin per 100 ml. This buffer was previously shown to remove >92% of cell surface-associated DNA from cells incubated at 4°C. The cell pellet was solubilized in 1 N NaOH and counted; a small aliquot was subjected to precipitation with 5% trichloroacetic acid (TCA) (final concentration). Identical experiments were performed in the presence of (*a*) 0.1 μg/ml of cycloheximide, (*b*) cells incubated at 4°C, and (*c*) HBSS plus 10 mM EDTA at 4°C.

In the immunofluorescent studies PBMC were incubated at 37°C with B-DNA (no azide present) for 2 h (HBSS + 1% BSA). Thereafter, the cell pellet was washed three times with 5 ml of HBSS plus 1% BSA and exposed to (*a*) goat anti-biotin, 30 min, washed three times; (*b*) FITC-labeled rabbit anti-goat, 30 min, washed three times; (*c*) Evans blue (0.005%) for 30 s, washed three times, and mounted with 50% glycerol in PBS. The slides were examined using epifluorescence from a mercury vapor lamp and a BG12 exciter filter and a 490-mm barrier filter. Photographs were taken using ASA 1000 film (Eastman Kodak Co., Rochester, NY), monitoring exposure time by using an Orthomat automatic light meter (E. Leitz, Inc., Rockleigh, NJ). Control experiments were: (*a*) cells incubated at 4°C in the presence of 15 mM sodium azide, (*b*) cells treated with trypsin (500 μg/ml, 37°C, 45 min), (*c*) cells exposed to a 50-fold excess of "cold" (i.e., nonbiotinylated) DNA before incubation with the B-DNA probe, and (*d*) omission of the B-DNA incubation step.

Results

DNA binding to intact cells. The integrity of the ^{3}H-labeled lambda phage DNA is shown in Fig. 1. We observed that with the tritium label, degradation does not occur under the conditions used (low DNAase concentration, short incubation), whereas the iodine-labeled DNA is significantly degraded. Sedimentation studies showed that the [^{3}H]DNA banded at 30% sucrose, corresponding to an $S_{20,w}$ of 34.9; the calculated $S_{20,w}$ for lambda phage DNA is 35.2. The binding of DNA to cells was maximal after incubation for 10 min at 4°C; it was dependent upon the presence of Ca, Mg, and SO_4 ions (Table I). It was abolished by proteolytic digestion of the cell surface but not by neuraminidase, phospholipase, or RNase (Table II). In an experiment to assess the effect of potential competitive inhibitors, only DNA and heparin influenced the binding of [^{3}H]DNA; transfer RNA poly [d(A)·d(T)], and a mixture of mononucleotides did not have any effect (Table III). [^{3}H]DNA could be

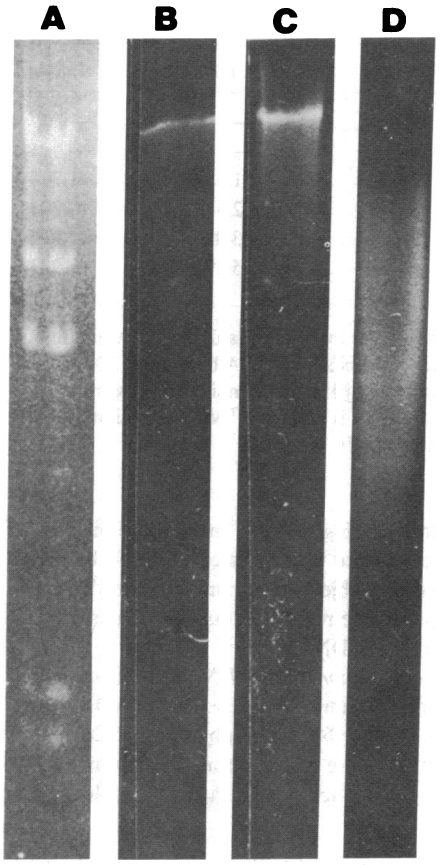


Figure 1. Electrophoresis in a 1% agarose gel of a Hind III digest of lambda phage DNA (lane *A*), ^{3}H-labeled DNA (lane *B*), unlabeled [^{3}H]DNA (lane *C*), and ^{125}I-labeled DNA (lane *D*). It is seen that the ^{3}H-labeled DNA is not degraded, whereas labeling with ^{125}I does result in significant degradation. Visualization is with ethidium bromide and ultraviolet fluorescence.

*Table I. Effect of Essential Ions upon DNA Cell Binding**

Concentration	Ca^{++}	Mg^{++}	$SO_4^{=}$
mM	*cpm*	*cpm*	*cpm*
0.0	58	18	16
0.01	672	761	720
0.10	893	943	916
0.50	1,092	1,073	1,163
1.00	1,301	1,262	1,325

$CaCl_2$, $MgCl_2$, and Na_2SO_4 were dissolved in Tris-HCl (0.15 M, pH 7.4). For each of the three ions, the concentration of two was kept constant at 1 mM, and the other *one* varied as shown under the concentration column. A standard [^{3}H]DNA binding assay was performed on cells suspended in the various ionic combinations. The results are expressed as the counts per minute of specific [^{3}H]DNA binding to 2 $\times 10^6$ neutrophils. It is seen that all three ions are necessary for DNA binding, with a critical range between 0.01 mM and 0.5 mM. The concentration of these ions in HBSS is: Ca^{++}, 0.7 mM; Mg^{++}, 0.9 mM; and $SO_4^{=}$, 0.4 mM; but it is seen that [^{3}H]DNA binding in HBSS is some nine times higher than the supplemented Tris-HCl buffer.
* Control HBSS, 11,981 cpm.

Table II. Effect of Enzymatic Digestion upon DNA Cell Binding

Treatment	DNA bound/2 $\times 10^6$ ADMC
	μg
Control	0.45
Pepsin (500 μg/ml)	0.42
Trypsin (500 μg/ml)	0.002
Protease (500 μg/ml)	0.001
Neuraminidase (10 μg/ml)	0.47
Phospholipase (200 μg/ml)*	0.39
RNAase (100 μg/ml)	0.41

Enzymatic treatment of cells (2 $\times 10^6$) was performed at 37°C for 1 h in HBSS/Hepes (pH 7.4). The cells were washed twice (4°C, 1 h) and a standard [^{3}H]DNA binding assay was performed.
* Enzymatic digestion at a pH of 5.6.

eluted from the cell surface by incubation at 4°C for 30 min with a 0.2-M glycine buffer, pH 2.8. DNA did not bind to erythrocytes, but the binding to all four other cell types (PMN, E−, E+, and ADMC) demonstrated saturability at DNA concentrations of ~600–800 μg/ml per 10^7 cells when nonspecific binding was accounted for (Fig. 2). A 50-fold excess of cold DNA reduced the binding by ~80%, and these figures were used to assess specific binding in the subsequent Scatchard analysis. Tryptic digestion reduced binding by ~90% over all values of added DNA in a curve paralleling the excess DNA curve (not shown in Fig. 2). Omission of the initial DNAse digestion step resulted in a reduced [^{3}H]DNA binding of ~20%, which suggested partial saturation of the DNA binding site. Scatchard plot analysis of the binding curves indicated that all four cell types displayed a high affinity (~10^{-9} M) for DNA binding; the data gave a straight line, which indicates a single population of binding sites (Fig. 3). Under these conditions the maximal binding of ^{3}H-lambda phage DNA to each cell type (molecules/cell) was: neutrophils, 2.6 $\times 10^3$; ADMC, 2.2 $\times 10^3$; E+ cells, 1.4 $\times 10^3$; and E− cells, 0.81 $\times 10^3$; these results are representative of eight such studies, with the interexperimental variation being <16%. As prior exposure to a specific ligand has been shown to influence several other ligand-receptor systems in a cooperative manner, PMNs were pulsed with cold DNA and then assayed for [^{3}H]DNA binding after various time periods of incubation at 37°C. We observed that after the expected inhibition of binding at zero time, normal binding was restored after ~2 h and increased thereafter in a manner consistent with "up-regulation" (Table IV).

Partial characterization of cmDNA and its binding to neutrophils. The binding of lambda phage DNA to human cells represents a rather artificial situation—a result of the ready availability of a dsDNA of well defined molecular weight. It is possible that the binding of DNA that was extracted from the surface of human cells might behave differently, due to variables such as size and affinity. To test this possibility, binding studies were performed using the DNA extracted from a purified cell membrane preparation from human neutrophils. The cmDNA sedimented in a linear sucrose gradient (5–35%) with a homogeneous peak at 19% concentration. For the conditions used (SW 50.1 rotor, 28,000 rpm, 17 h, 4°C) this corresponds to a sedimentation coefficient of 18.1 S, or a molecular weight of 4.8 $\times 10^6$, calculated using the equation of Studier (26a). The peak

Table III. Effect of Potential Inhibitors of DNA Cell Binding

Inhibitor	Zero	× 0.5	× 1.0	× 2.0	× 10	× 100	× 1,000
Control (no inhibitor)	12,796	—	—	—	—	—	—
DNA	—	1,076	908	348	—	—	—
tRNA	—	—	—	—	11,578	11,434	11,793
Poly [d(A)·d(T)]	—	—	—	—	12,867	12,459	10,201
dCMP, dGMP, dAMP	—	—	—	—	13,074	13,026	12,006
Heparin	—	—	—	—	8,555	5,191	241

Neutrophils (2×10^6) were preincubated with the potential competitor for 15 min at 4°C, and then subjected to the standard [^{3}H]DNA binding assay, without removal of the competing substance. The results are expressed as the counts per minute of specific [^{3}H]DNA binding to 2×10^6 neutrophils. The first row refers to the multiplication factor for each competitor, using the saturation level of [^{3}H]DNA binding (800 μg/ml, or $\sim 2.5 \times 10^{-8}$ M) as the base-line concentration; e.g., × 10 for RNA indicates that the concentration of tRNA is 2.5×10^{-7} M. The inhibition of binding by 2.5×10^{-5} M heparin could be reversed by washing the cells twice before incubation with [^{3}H]DNA.

of ^{3}H counts corresponded to the OD_{260} peak of unlabeled cmDNA, which indicates absence of degradation during the process of nick translation labeling. Our cmDNA may have some tenacious protein impurities, as the best $OD_{260/280}$ ratio that we could obtain after experimentation with several different procedures for DNA extraction was 1.48. Characterization of strandedness indicated cmDNA to be >90% dsDNA on hydroxyapatite chromatography with 95% of molecules possessing short single-stranded regions as assessed by chromatography on benzoylated-naphthoylated DEAE cellulose chromatography (27). The affinity (K_d) for cmDNA binding to neutrophils was 1.9×10^{-8} M, and 4.3×10^4 molecules were maximally bound; compared with lambda phage DNA, this represents a decreased affinity of about one order, but represents an increase in the number of molecules bound.

DNA binding to cell membranes. Bearing in mind that whole cells contain many potential DNA binding proteins in the nucleus, we studied the binding of DNA to purified cell membrane preparations from each cell type. Consistent with the results of the cell-binding experiment, DNA sedimented with the intact cell membrane preparation (Fig. 4). Cell membrane binding of DNA was essentially abolished by prior treatment of the membranes with trypsin, or by subjecting the membrane-[^{3}H]DNA complex to deoxycholate; the released counts sedimented in a position consistent with free DNA.

Preliminary characterization of a cmDNA binding molecule. Purified membrane preparation of E−, E+, PMN, ADMC, and RBC as well as cytosol and NHNP were subjected to SDS-PAGE and the separated proteins were electrophoretically blotted onto nitrocellulose sheets. A modification of the methodology used

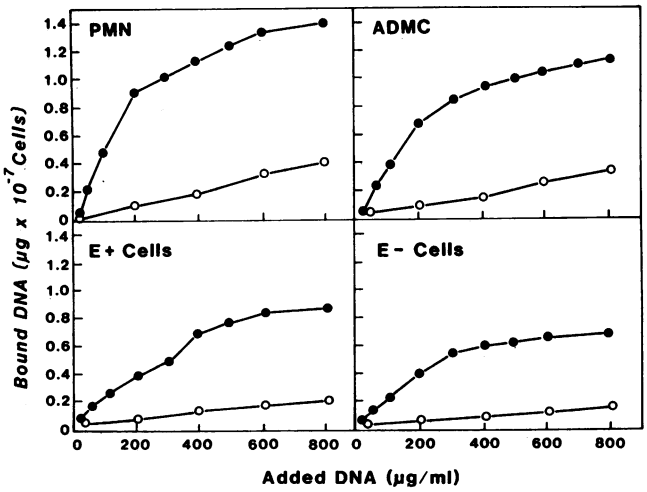


Figure 2. Cells were processed as per the flow sheet in Scheme I. It is seen that for each cell type a saturability of [^{3}H]DNA binding is demonstrated. Untreated cells, •; excess cold DNA, o. Trypsinized cells: points not shown, exhibited a parallel curve to the "excess cold DNA" curve.

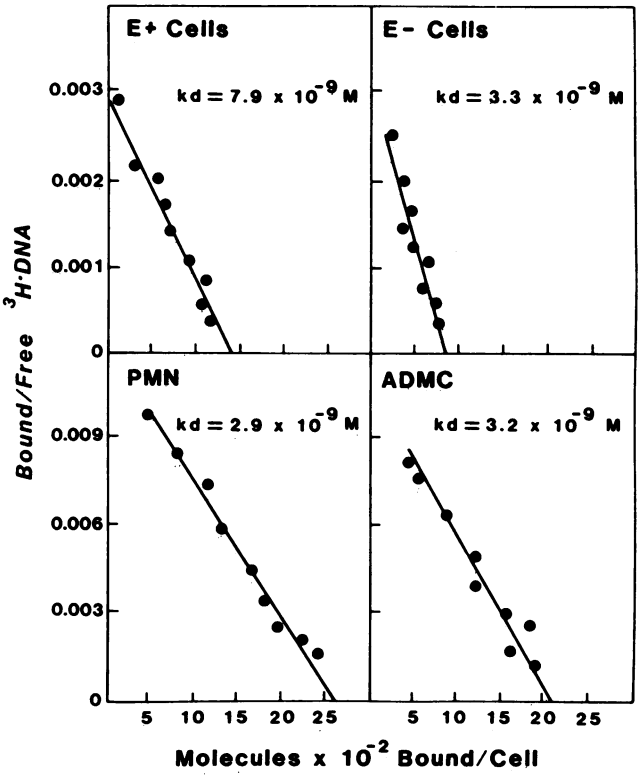


Figure 3. A linear transformation of the data shown in Fig. 1 to yield Scatchard plots. For each cell type a straight line relationship was observed ($r^2 > 0.89$), indicating a single species of binding molecule. The dissociation constants (K_d) were $\sim 10^{-9}$ M, and the DNA binding capacity varied from 0.81×10^3 to 2.6×10^3 molecules per cell, depending upon the cell type.

Table IV. Modulation of [^{3}H]DNA Binding by Prior Exposure to Cold DNA

Time after cold DNA pulse	DNA bound/2 × 10^6 PMNs
h	*μg*
Zero	0.006
1	0.41
2	0.78
3	0.97
4	1.12
18	1.80
Control at 18 h (no DNA pulse)	0.68
Basic binding (no DNA pulse, incubation for 1 h at 4°C)	0.47

PMNs (2 × 10^6 for each time point) were incubated with 800 μg of cold DNA for 1 h, 4°C. After being washed three times, the cells were incubated at 37°C and at the time intervals shown, a standard [^{3}H]DNA binding assay was performed. In one control the PMNs, incubated for 18 h at 37°C, were not pulsed with cold DNA; in another control the basic binding at 4°C was assessed.

by Bowen et al. (25; and Methods) for identifying DNA binding proteins in nuclear extracts was used to probe for DNA binding proteins in cell membrane preparations; the modification described in this communication employs B-DNA instead of [^{3}P]DNA. A single band of B-DNA binding was observed at the same position in preparations from E+, E−, ADMC, and PMN; *no* B-DNA binding was observed with the RBC membrane preparation (Fig. 5). As a positive control, nuclear proteins were seen to show several bands of DNA binding, including a band

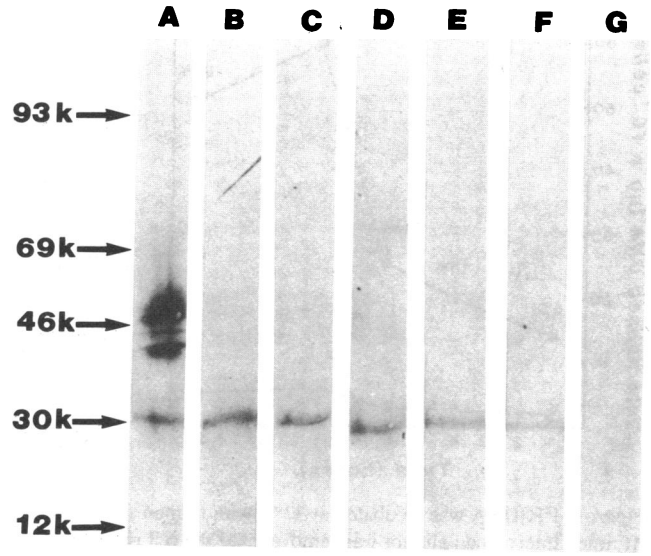


Figure 5. Nonhistone nuclear proteins (*A*), membrane preparation of neutrophils (*B*), monocytes (*C*), T lymphocytes (*D*), B lymphocytes (*E*), a cytosol preparation (*F*), and erythrocyte membranes (*G*), were subjected to SDS-PAGE. The separated proteins were electrophoretically blotted onto nitrocellulose sheets; DNA binding proteins were probed for by biotin-DNA. Development was accomplished with a streptavidin-biotinylated horseradish peroxidase complex using the substrate diamino-benzidine tetrahydrochloride. All cells, except erythrocytes, possessed a single DNA binding protein, migrating in a position consistent with a molecular weight of 30,000. Cytosol exhibited a weak binding band. Nonhistone nuclear proteins are included as a positive control and also show a DNA binding protein in an identical position to the cell membrane preparations.

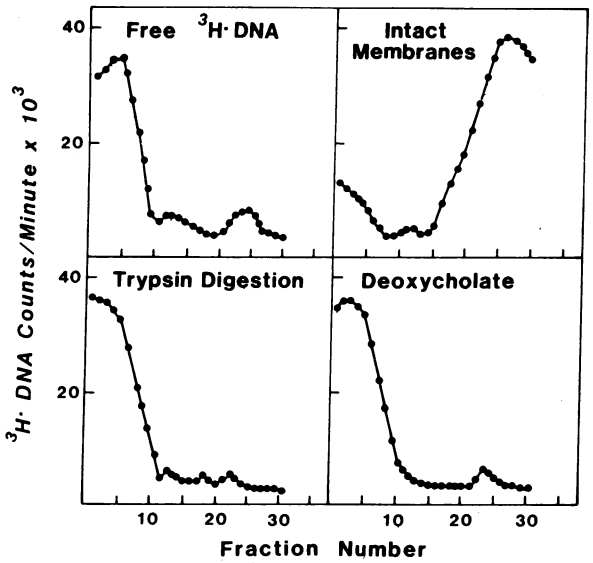


Figure 4. A purified membrane preparation from human neutrophils was incubated with ^{3}H-lambda phage DNA and subjected to rate zonal sedimentation (SW 20 rotor, 10–45% sucrose, 100,000 *g*, 3 h, 4°C). Fractions were collected from the top downward and the radioactive peaks compared with free [^{3}H]DNA. Membranes previously exposed to trypsin did not bind [^{3}H]DNA, whereas a [^{3}H]DNA membrane complex released free [^{3}H]DNA after disruption with deoxycholate.

in an identical position with the cmDNA binding protein. A cytosol preparation in a concentration threefold greater than the cell membrane preparations shows a very faint band of DNA binding. To determine whether the binding protein was reactive with biotin, rather than DNA, we performed the same procedure, using ^{125}I-DNA as the probe with visualization by autoradiography. Preparations from PBMC, neutrophils, and ADMC, but *not* RBC, exhibited an identical band of ^{125}I binding in a similar position to the bands visualized with the B-DNA. The estimated molecular weight of the membrane-associated DNA binding protein was 30,000. Prior trypsinization of the cell membranes, and the omission of the B-DNA incubation step abrogated the development of the DNA binding band.

Internalization and degradation of DNA. When monocytes were incubated over a 25-h period at 37°C in HBSS in the continuous presence of [^{3}H]DNA, there was a progressive increase in the amount of [^{3}H]DNA that was not susceptible to acid elution from the cell surface (Fig. 6). Whereas the glycine wash maintained a TCA precipitability of >85%, there was a progressive increase in the TCA-soluble fraction associated with the cell pellet; at 25 h only 19% of the internalized DNA was insoluble in TCA (5% TCA precipitates oligonucleotides > 10–12 nucleotides). The inclusion of cycloheximide (an inhibitor of ribosomal protein translation) in the culture medium resulted in minimal internalization of DNA. When cells were incubated at 4°C the amount of internalized DNA at 25 h was ~12 μg per 10^7 cells (data not shown). When the culture medium was supplemented with 10 mM EDTA, no uptake and internalization

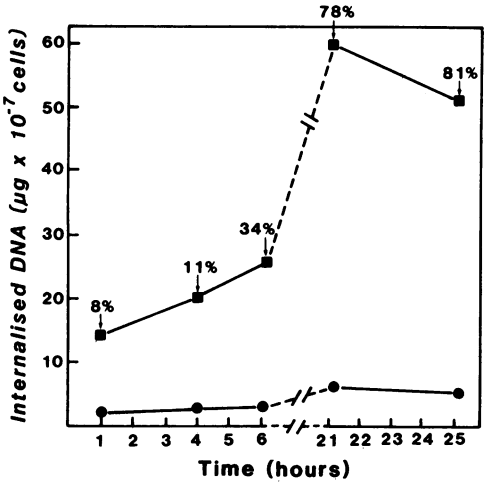


Figure 6. [^{3}H]DNA was incubated at 37°C with human monocytes. At timed intervals an aliquot was removed and the cell pellet washed with a 0.2-M glycine buffer, pH 2.8, to remove surface associated [^{3}H]DNA. The cell pellet was solubilized in 1 N NaOH and its radioactivity was counted; a small aliquot was subjected to precipitation with 5% TCA. It is seen that there is a progressive internalization of [^{3}H]DNA up to ~21 h (■), with an associated degradation of DNA, as assessed by TCA solubility (arrows indicate percentage of TCA-soluble material). Cells incubated with cycloheximide (●) showed virtually no internalization of [^{3}H]DNA.

was observed; this result is in keeping with the previous observation indicating that calcium and magnesium ions are essential for the DNA-receptor interaction.

So that we could directly visualize the binding of DNA to intact cells and observe its fate on incubation, we used biotinylated DNA as a probe (as outlined in Methods). ~65% of the cells incubated at 4°C in the presence of azide exhibited a circumferential staining pattern (Fig. 7 *A*). Prior treatment of the cells with trypsin abolished specific binding as did excess cold DNA and the omission of the incubation step with B-DNA. When cells were incubated at 37°C, a redistribution of the surface fluorescence and capping occurred over a time interval of ~30–45 min (Fig. 7 *B* and *C*). After ~2 h some cells exhibited internalization of DNA with an apparent localization to intracytoplasmic vacuoles (Fig. 7 *C*).

Discussion

There is accumulating evidence that nucleic acids are found in association with cell surfaces (8–11, 17). The present study presents evidence that such an association may in some instances be mediated by a specific surface binding site. The source of the DNA apparently bound to certain peripheral blood cells (17) remains a controversial issue; a widespread notion is that it originates from dying cells (16). On the other hand there is abundant evidence that stimulated lymphocytes release DNA (1, 3–6) and these could be another source of cmDNA. Rogers (28) has presented evidence to indicate that phytohemagglutinin-stimulated peripheral blood lymphocytes secrete DNA, which subsequently becomes bound to the cell membrane and is actively capped. This observation lends some support to our present findings of an apparent receptor for DNA and is in accord with the earlier work of Olsen and Harris (29) and Ehrlich et al. (30), which indicated the likelihood that mammalian cells could sequester exogenous DNA.

The current findings regarding the saturability of the binding sites, its dependence upon calcium ions, the dissociation constant of 10^{-9} M, and the inhibition of [^{3}H]DNA binding by excess cold DNA, but not RNA or other similar molecules, fulfill most of the conditions generally accepted as being consistent with a specific receptor (31). Further evidence for a receptor-mediated process comes from the demonstration that surface adherent DNA is internalized and degraded to small oligonucleotides. This phagocytic process occurs over a fairly extended time span, with maximal uptake occurring after ~18 h, and is inhibited by the presence of cycloheximide. Over this time course, approximately 40 times more molecules are internalized than can be shown to bind to the cell surface at maximum saturation. These features are most consistent with the notion that after binding to the receptor, the DNA-receptor complex is internal-

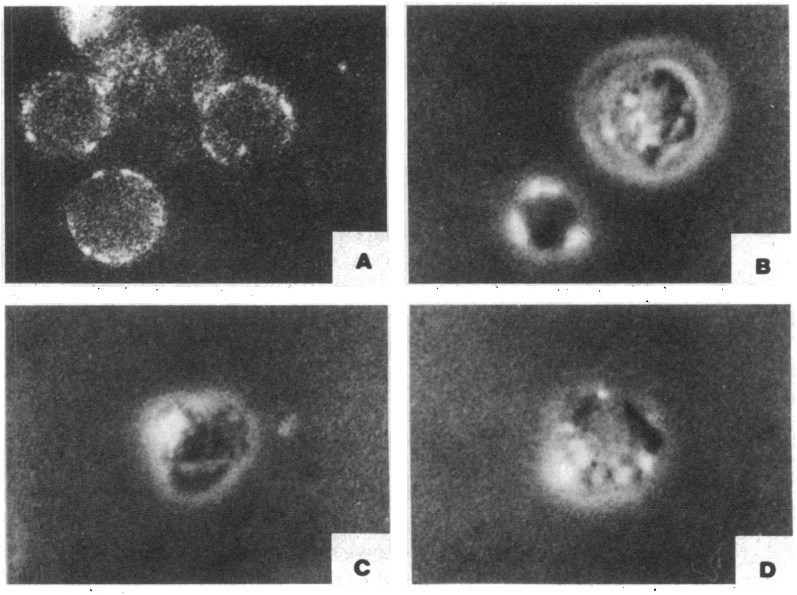


Figure 7. Indirect immunofluorescence (using FITC rabbit anti-goat and goat anti-biotin) of human peripheral blood mononuclear cells that have been incubated with biotin-labeled DNA. (*A*) Incubation at 4°C: three cells are seen with a pattern of peripheral staining and one cell with diffuse staining (presumably dead). (*B*) Incubation at 37°C, 45 min. Two cells are seen; one cell, probably a small lymphocyte, shows a redistribution of surface fluorescence into three patches; the other cell, probably a monocyte, shows internalization of DNA. (*C*) Incubation at 37°C, 45 min: a single cell is seen exhibiting a "capping" of the surface fluorescence. (*D*) Incubation at 37°C, 2 h: a single cell is seen with an appearance of intracytoplasmic vacuoles containing internalized DNA.

ized and both the receptor and DNA are degraded, with subsequent re-expression of the receptor depending upon active ribosomal translation. This interpretation implies that the DNA receptor is a regenerated receptor rather than a recycled receptor. The immunofluorescent studies indicate that surface redistribution of DNA and capping occur before its internalization into discrete intracytoplasmic vacuoles; this phenomenon does not occur at 4°C in the presence of sodium azide. These events are compatible with a process of adsorptive endocytosis dependent upon a ligand-directed redistribution of DNA receptors into coated pits (32). Whether the internalization and degradation of DNA subserves a critical physiological function remains to be determined. It would seem probable that the DNA receptor could act as a scavenger for circulating DNA, effecting its removal from the blood stream. There is solid evidence to implicate the liver as the major site for the removal of circulating DNA (33), and it will be of interest to determine whether hepatic reticuloendothelial cells possess a DNA receptor.

There are several technical aspects of this study that deserve further comment. The values for the maximal number of DNA molecules bound per cell should not be interpreted as being equivalent to the total number of receptor sites. It is apparent from the experiment with a relatively low molecular weight DNA, isolated from the cell surface of a normal neutrophil donor, that the number of receptor sites for DNA will probably be in excess of 5×10^4. This figure may be in error as the $OD_{260/280}$ ratio of the cmDNA was only 1.48, which suggests protein impurities. Despite several different DNA extraction techniques we have been unable to isolate cmDNA with a higher $OD_{260/280}$ ratio. It may be that cmDNA has an unusual G + C content; on the other hand, a contaminating DNA binding protein could lead to aggregation and erroneous sedimentation characteristics. We believe this is unlikely, as the calculated molecular weight of this DNA was 4.8×10^6. This value is in close agreement with the studies of Meinke et al. (11), who found cmDNA isolated from diploid human lymphocytes to have a molecular weight of 4.2×10^6. Furthermore, the K_d of 10^{-9} M, found for lambda phage DNA, probably represents a higher affinity than will be found for a small DNA molecule binding to a single receptor; it is very likely that a large DNA molecule will attach to multiple binding sites, and in doing so, its apparent dissociation constant will be enhanced. We are currently exploring this hypothesis using well defined DNAs of varying sizes. Another source of error in the results reported here stems from the modulation of binding capacity by incubation at 37°C for 18 h and the apparent "up-regulation" by prior pulsing of cells with cold DNA (see Table IV). It is conceivable that a portion of cmDNA attached to a specific binding molecule is not accessible to the DNAase used to denude the cells before our binding experiment. If this were so, the DNA-receptor complex may be internalized, and newly synthesized unoccupied receptors may be expressed at the cell surface, thus making extra binding sites available after a finite period of time. It is of some technical interest that when we used very low concentrations of DNAase 1 (100 pg/ml), no degradation was observed with ^3H labeling, whereas considerable degradation was present in the ^{125}I-labeled DNA. This is in keeping with the observation of Mackey et al. (34), who noted less breaks in nick-translated DNA labeled with ^3H compared with ^{32}P, and also noted an inverse correlation between fragment size and DNAase concentration. Absence of DNA degradation is of course essential in the analysis of binding studies in order to obtain an accurate estimation of the affinity and maximal number of molecules bound. Although binding of [^3H]DNA to intact cells is dependent upon the presence of low concentrations of Ca, Mg, and SO_4 ions, such ions were not present in the buffer used to dissolve the B-DNA probe. The discrepancy between the requirements for binding DNA to intact cells, vs. the binding to proteins resolved by SDS-PAGE, cannot be explained currently; however, boiling proteins in SDS and then attempting to renature them on nitrocellulose sheets may well result in conformational changes that could modify their DNA binding properties.

Confirmation of our findings concerning an apparent receptor for DNA could have implications for molecular biologists concerned with the introduction of genomic DNA into cells, as well as rheumatologists and immunologists involved in unravelling the phenomenon of DNA-anti DNA interrelations in systemic lupus erythematosus.

Acknowledgments

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