Virchows Arch (2000) 437:380–387

ORIGINAL ARTICLE

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Impairment of genomic DNA binding to a putative dysfunctional receptor on erythrocytes independent of complement and antibodies in systemic lupus erythematosus

Received: 2 May 2000 / Accepted: 9 May 2000

Abstract Systemic lupus erythematosus (SLE) is characterized by a variety of autoantibodies and other immune abnormalities indicative of an immunological hyperactivity. Antibodies against native DNA, however, are a disease-specific marker and play a major role in the pathogenesis of systemic or organ-specific disease manifestations. Nevertheless, the mechanisms causing the appearance of autoantibodies and immune complexes in SLE are not yet understood. Here, we report that chromosomal DNA and other forms of nucleic acids are usually cleared from circulation by binding to a yet unidentified receptor-like protein on the surface membrane of erythrocytes, independently from complement or antibodies. The binding kinetics of DNA and other nucleic acids to erythrocytes are significantly altered in SLE patients, showing an overall reduced binding capability and presaturated binding kinetics. Significant amounts of chromosomal DNA can be isolated from erythrocytes of SLE patients but not from normal controls. Electron microscopy shows electron-dense particles on the surface of SLE erythrocytes (approximate size 20–40 nm). Comparative genomic hybridization reveals that the nucleic acid isolated from erythrocytes of SLE patients is of genomic and random origin, leading to an accumulation of "free" nucleic acids in the periphery, which eventually induces a B-cell immune response.

Keywords Systemic lupus erythematosus · DNA · Erythrocytes · Receptor

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Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by anti-DNA autoantibodies and circulating immune complexes (CICs), consisting of these autoantibodies and mostly double-stranded (ds) DNA [19]. The persistence of CICs in the periphery leads to a broad immune response and eventually to the typical pattern of clinical symptoms affecting many different organ systems [35]. Cell death, occurring physiologically at any time in almost all proliferating organ systems, may lead to the liberation of nucleic acids from the cell [4]. From studies of cells undergoing apoptosis, it is known that the nucleus-derived DNA is cleaved into pieces of various sizes [8]. To avoid any danger caused by a prolonged persistence of nucleic acids in the circulation, various humoral and cellular mechanisms are involved in the clearance of the nucleic acids [1, 28]. Besides non-specific cleavage of circulating DNA by plasma nucleases (e.g., DNase I) and clearance by the reticulo-endothelial system (RES) of the liver and spleen [10], macrophages and monocytes usually phagocytize fragments of the nucleus and histone–DNA complexes [3, 6, 7]. An alternative clearance mechanism for nucleic acids from peripheral blood involves red blood cells and the complement receptor CR1 [9, 18]. CICs can be bound to the CR1 on erythrocytes via the complement factor C3b, accelerating the RES-mediated phagocytosis [23, 29, 30]. If DNA clearance systems fail to eliminate nucleic acids, persistently circulating nucleic acids eventually become immunogenic and can lead to a humoral immune response and the production of anti-DNA antibodies [17, 25, 32].

Although the etiopathology of SLE is yet unknown, the characteristic features of the disease suggest that there is a breakdown in normal immune surveillance mechanisms. There appear to be many factors that predispose the development of SLE. Other than speculations on the possible role of viruses [15], hormonal and genetic factors seem to cause a predisposition for the disease, or at least for disease progression. There is a female pre-

disposition for SLE, occurring 9:1 in women between the age of 12 years and 40 years [26]. There is also a genetic predisposition, reflected by a higher incidence of SLE in families with monozygotic twins. The disease is correlated with the expression of certain major histocompatibility complex (MHC) class-II molecules [human leukocyte antigen (HLA)-DR2 and HLA-DR3] [12] and a deficiency of the complement factors C1q, C2, and C4 [11]. All of these predisposing factors would also contribute to a defective clearance of plasma nucleic acids and a loss of normal immune self-tolerance. Here, we investigate the complement and antibody-independent clearance of nucleic acids from circulation and the possible origin of erythrocyte-bound nucleic acids in SLE patients.

Materials and methods

Blood samples

Heparinized blood was obtained from patients diagnosed with SLE at the Department of Medicine III, University of Erlangen-Nürnberg and the Childrens' Hospital of the University of Munich (Dr. von Haunersches-Kinderspital). Most of the patients were female (n=12), although some male blood samples could be collected (n=4). Control blood was obtained from healthy volunteer donors of both gender (n=17).

Isolation of plasma nucleic acids

As described elsewhere, nucleic acids were isolated from the plasma of SLE patients [21] and used for binding studies in parallel with other nucleic acids, such as ds lambda-phage DNA, salmon sperm DNA, RNA, and polyguanosin (Sigma Aldrich).

Iodine labeling of nucleic acids

Various commercially available nucleic acids (lambda-phage DNA, salmon sperm DNA, RNA, polyguanosin) and SLE plasma-derived nucleic acids were radiolabeled using $Na^{125}I$ (Amersham). Briefly, 0.5 µg of the nucleic acids were incubated with 10 µl buffer (0.475 mM tallium-III-nitrate, 4.2 mM HNO_3, and 62.5 mM ammonium sulfate) and 4 µl $Na^{125}I$ (1400 Ci/mM) for 5 min at 64°C. To eliminate free $Na^{125}I$, the nucleic acids were precipitated over night in ethanol at –80°C, and the precipitate was dissolved in phosphate-buffered saline (PBS).

Density gradient of heparinized peripheral blood

Heparinized blood was incubated with various radiolabeled nucleic acids. The incubation time varied from 5 min to 30 min. After incubation at 37°C, a two-step density gradient was established using Ficoll-Hypaque (Histopaque 1077, Sigma, Mo.) at concentrations of 69% (d=1.053) and 61% (d=1.047). On top of this gradient, heparinized blood was overlayed and centrifuged for 20 min at 400×g without a brake. Six different layers of cellular and soluble fractions were carefully removed and placed into separate vials. The specific activity was measured in an automated gamma counter (Coulter). Only trichloric-acidic acid precipitable activity was evaluated.

Spectrophotometric analysis of DNA

A pure erythrocyte suspension (10 µl) from SLE patients and normal controls was dissolved in 2 ml isotonic NaCl and incubated with different concentrations of lambda-phage DNA (0.5–4.0 µg) for 5 min at 37°C. The suspension was then centrifuged for 10 min at 400×g, and 200 µl of the supernatant was removed. The DNA concentration in the supernatant was determined using 2'-4'-diamidinophenylindole (DAPI) and spectrophotometry (fluorometry) as described elsewhere (MPF-3L; Perkin-Elmer) [20]. To investigate the various factors that might influence the binding kinetics of DNA to erythrocytes, we pre-incubated the cells with heat-inactivated serum or bovine serum albumin (0–60%). In addition, different pHs were used from 7.2 to 7.6. The incubation time was also varied and extended up to 2 h.

Cold target inhibition (binding kinetics)

Erythrocytes of normal controls were incubated with 1 µg radiolabeled lambda-phage DNA for 5 min. The erythrocyte suspension was thoroughly washed and incubated with various concentrations of unlabeled DNA for another 10 min. Erythrocytes were then washed again, and the specific erythrocyte-bound radioactivity was determined as described above.

Isolation of nucleic acids from erythrocytes

Erythrocytes from SLE patients and normal controls were purified. Contamination with nucleated cells was excluded. Erythrocytes were lysed using ammonium chloride, and the membranes were digested with proteinase K for 24 h at 50°C. The proteins of the cell lysate were then denatured using phenol/chloroform, and nucleic acids were precipitated using two volumes of ethanol (–80°C, overnight). The ethanol precipitate was re-dissolved in PBS, and 20 µl DNA solution was run on an agarose gel (0.8%). An aliquot of the solution was preincubated with the restriction enzyme *Hae*III for 30 min at 37°C prior to gel electrophoresis. DNA from mononucleated cells (MNCs) was also isolated and electrophoresed as a control.

Comparative genomic hybridization

To identify the origin of the SLE antigen (DNA), we performed a comparative genomic hybridization (CGH) using nucleic acids isolated from erythrocytes of SLE patients. The nucleic acids were amplified and directly labeled by means of degenerate oligonucleotide primer (DOP)-polymerase chain reaction (PCR) as described elsewhere [22]. The fluorescein-labeled SLE DNA and the Texas Red-labeled control DNA were hybridized to normal lymphocyte metaphases to locate specific regions of the human genome that might represent chromosomal regions, preferentially bound to patients' erythrocytes.

Electron microscopy of erythrocytes

For transmission electron microscopy (TEM), erythrocytes were fixed in Karnovsky/Karnovsky 2 and medium (1:1), washed in 2% OsO_4 for 1 h, and dehydrated in ethanol at increasing concentrations for 15 min. Preparations were incubated again in Karnovsky/Karnovsky 2 and medium and placed in 100% EPON 812 overnight. Embedding in fresh EPON 812 was performed by inverting plastic capsules filled with EPON 812 on appropriate areas and curing for 48 h in a 60°C oven. Preparations were then sectioned on a Porter-Blum MT-1 ultramicrotome with a diamond knife. For examination, Tsousinius 200 mesh grids were used. The preparations were double stained with saturated 6% uranyl acetate for 2 h, rinsed, exposed to Millonig's lead stain for 4 min, rinsed again, and examined with a JEOL 100 SX electron microscope.

Results

Distribution of nucleic acids in peripheral blood

The binding of ^{125}I-DNA (ds lambda-phage DNA) to peripheral blood cells after 5 min incubation at 37°C and

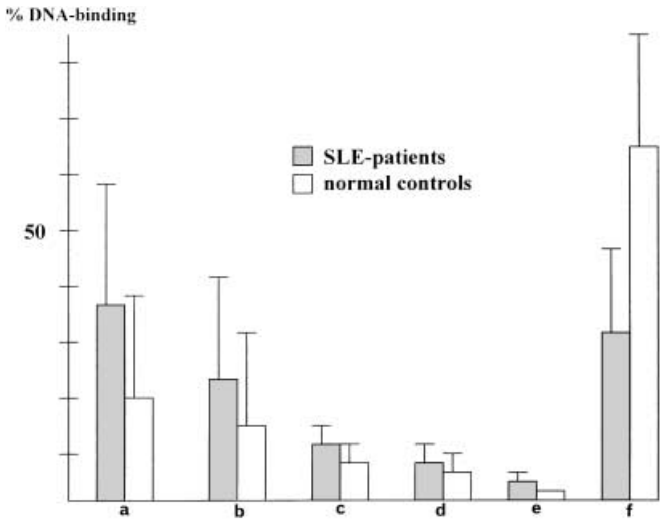

Fig. 1 Binding of ^{125}I-DNA [double stranded (ds) lambda-phage DNA] to peripheral blood cells after a 5-min incubation and centrifugation of 20 ml heparinized blood over a modified Ficoll-hypaque gradient. Total gamma activity was measured per fraction. Patients with systemic lupus erythematosus (SLE) (n=16) showed a significantly reduced binding of ^{125}I-DNA to erythrocytes compared with healthy controls (n=12; P<0.01). Any other differences were not significant. The binding pattern of other nucleic acids, such as salmon sperm DNA, RNA, or polyGuanosin (pG), were comparable with those of ds lambda-phage DNA and showed no significant differences. **a** Plasma; **b** mononuclear cells (~5×10^6 cells); **c** gradient; **d** granulocytes (~1×10^7 cells); **e** interphase; and **f** erythrocytes (1×10^{10} cells)

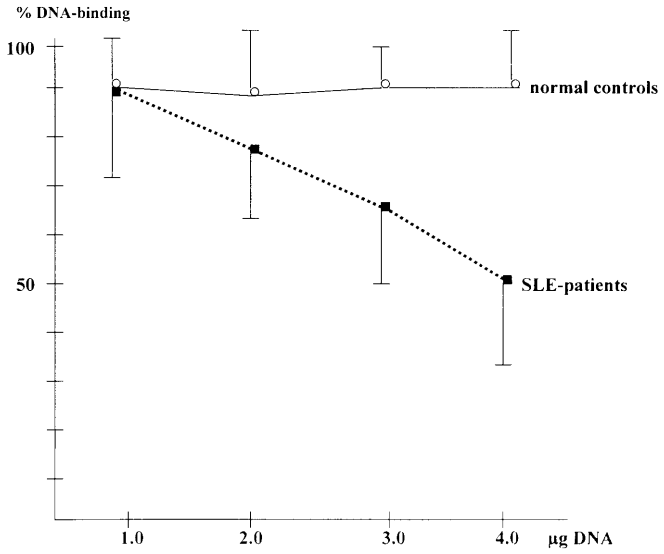

Fig. 2 Binding of double stranded (ds) DNA to a 10% erythrocyte preparation. Nucleic acid (1.0–4.0 µg) was incubated with 100 µl erythrocyte suspension for 5 min at 37°C. After centrifugation, unbound DNA was quantified in the supernatant using spectrophotometry and DAPI (2'-4'-diamidinophenylindole) incorporation. While erythrocytes of normal control persons were able to bind almost all dsDNA, erythrocytes from patients with systemic lupus erythematosus (SLE) were early saturated and the binding ability decreased linearly with increasing dsDNA concentrations

centrifugation over a modified ficoll-hypaque gradient was analyzed. Total radioactivity was measured per fraction. We found that iodinated ^{125}I-dsDNA is bound predominantly by the erythrocyte fraction (53±8%) in SLE patients and in normal controls (Fig. 1f). Nevertheless, SLE patients showed a significantly reduced binding of ^{125}I-DNA to erythrocytes (32±11%) compared with healthy controls (64±15%). The binding patterns of other nucleic acids, such as salmon sperm DNA, RNA, or polyGuanosin (pG), were comparable with those of ds lambda-phage DNA and showed no significant differences. Additional precipitable activity (using trichloric-acidic acid) was found either in the MNC fraction (Fig. 1a) or in the plasma fraction (Fig. 1b). In these fractions, the specific activity was higher in SLE patients than in normal controls.

Comparison of DNA binding to erythrocytes from SLE patients and controls

Different amounts of nucleic acid (1.0–4.0 µg) were incubated with the purified erythrocyte suspension of SLE patients and normal controls for 5 min at 37°C. Erythrocytes of normal controls were able to bind almost all dsDNA (92±8%) at this DNA–erythrocyte ratio, while erythrocytes of SLE patients were already saturated with 1 µg dsDNA. The binding ability of SLE erythrocytes decreased further with increasing concentrations of nucleic acids (Fig. 2). The preincubation of erythrocytes with serum or bovine serum albumin (BSA), the change of the pH, and an extension of the incubation time up to 2 h had no influence on the binding capacity.

Comparison of dsDNA and nucleic acids isolated from plasma of SLE patients

The binding ability of dsDNA (lambda-phage DNA; Sigma) was compared with those of nucleic acids isolated from SLE patients [15]. Both show a significantly reduced binding to erythrocytes of SLE patients, but nucleic acids isolated from the plasma of SLE patients did not bind as efficiently to erythrocytes when compared with control (commercially available) dsDNA (Table 1). This suggests that the nucleic acids isolated from the plasma of SLE might still contain significant amounts of proteins or contain structures that interfere with the DNA binding to erythrocytes [34].

Receptor binding kinetics

Erythrocytes of normal controls were preincubated with ^{125}I-DNA, washed, and then incubated with increasing amounts of unlabeled dsDNA for a competitive binding

Table 1 Binding of nucleic acids from different sources to normal erythrocytes and erythrocytes of systemic lupus erythematosus (SLE) patients

	dsDNA (lambda-phage)	Plasma nucleic acids (SLE)
Erythrocytes of SLE patients	26–38% (*x*=31%)	18–30% (*x*=24%)
Erythrocytes of controls	50–65% (*x*=61%)	35–45% (*x*=41%)

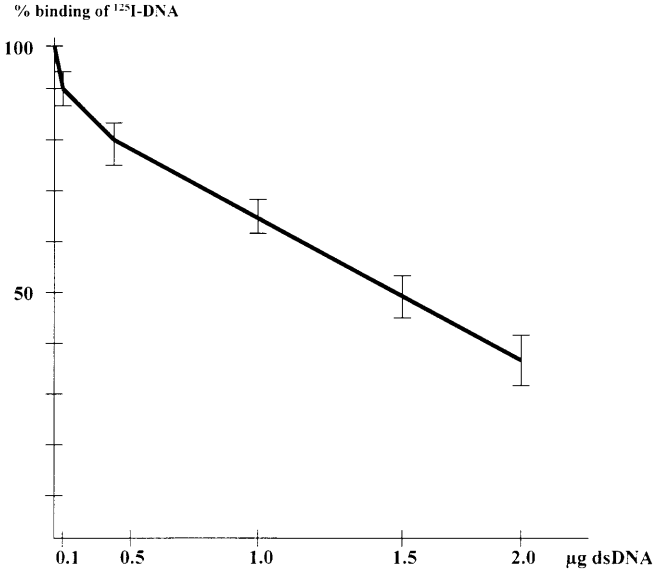

Fig. 3 Binding of ^{125}I-DNA to an erythrocyte suspension of normal controls. Erythrocytes were preincubated with radiolabeled ^{125}I-DNA and then incubated with increasing amounts of double-stranded (ds) DNA. The figure shows the typical receptor–ligand kinetics expected for a DNA–protein interaction. The prebound ^{125}I-DNA competes with dsDNA for a receptor-binding domain

study. Figure 3 shows the typical kinetics expected for a receptor–ligand interaction. The unlabeled dsDNA competes with ^{125}I-DNA for the DNA receptor-binding domain on the surface of erythrocytes, suggesting that there possibly exists a receptor-like domain, specifically binding DNA. This putative receptor may be functionally defective in SLE patients. This receptor binding study did exclude any participation of anti-DNA antibodies or complement in the receptor–ligand interaction.

Chromosomal DNA on erythrocytes

The isolation of DNA from erythrocytes of SLE patients revealed the presence of particular high-molecular weight DNA, which was similar in size to chromosomal DNA that was extracted from MNCs of SLE patients (approximate size 10 kb; Fig. 4). Pre-treatment of the DNA with the *Hae*III restriction enzyme yielded a complete digest, suggesting that there is no obvious enzyme-resistant sequence in the DNA bound to the surface of SLE erythrocytes. Erythrocytes of normal controls showed that no significant amounts of DNA could be detected using this method (not shown).

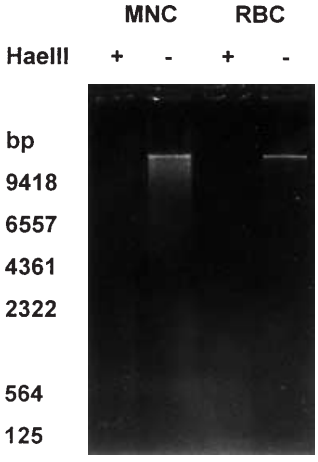

Fig. 4 The isolation of DNA from the erythrocytes of systemic lupus erythematosus (SLE) patients shows the presence of particular high-molecular weight DNA, which is similar to the high-molecular weight DNA that was extracted from mononuclear cells of SLE patients (approximate size 10 kb). Treatment with the *Hae*III restriction enzyme yielded a complete digest. Erythrocytes of normal controls showed no significant amounts of DNA (not shown)

Genomic origin of nucleic acid bound to erythrocytes in SLE

CGH of DNA, isolated from erythrocytes of SLE patients and hybridized on normal metaphases, showed no prevalence of certain chromosomal regions (Fig. 5), suggesting that the DNA bound to the erythrocyte surface might be random, genomic DNA. The red color labeling of the telomere and centromere regions is due to repetitive sequences and is therefore not evaluable [21].

Electron-dense particles on erythrocytes of SLE patients

Electron microscopy revealed the presence of electron dense particles on the surface of erythrocytes of SLE patients with an average size of 20–40 nm (Fig. 6A). Erythrocytes of normal controls did not show any such structures (Fig. 6B), but had the regular glycokalix structure.

Discussion

The deposition of immune complexes and the presence of CICs are probably the most pathogenic factors involved in the pathogenesis of SLE. In spite of intensive research activities over the past years to explain this autoimmune disease, there is still no comprehensive theory for all of the disease-associated phenomena.

Cell death can occur during a trauma with tissue damage, and nucleic acids are released into the interstitium of the blood stream. However, even smaller amounts of nucleic acids are released on a more regular basis during cell renewal and apoptosis. Usually, these nucleic acids do not induce any immunological reaction because they

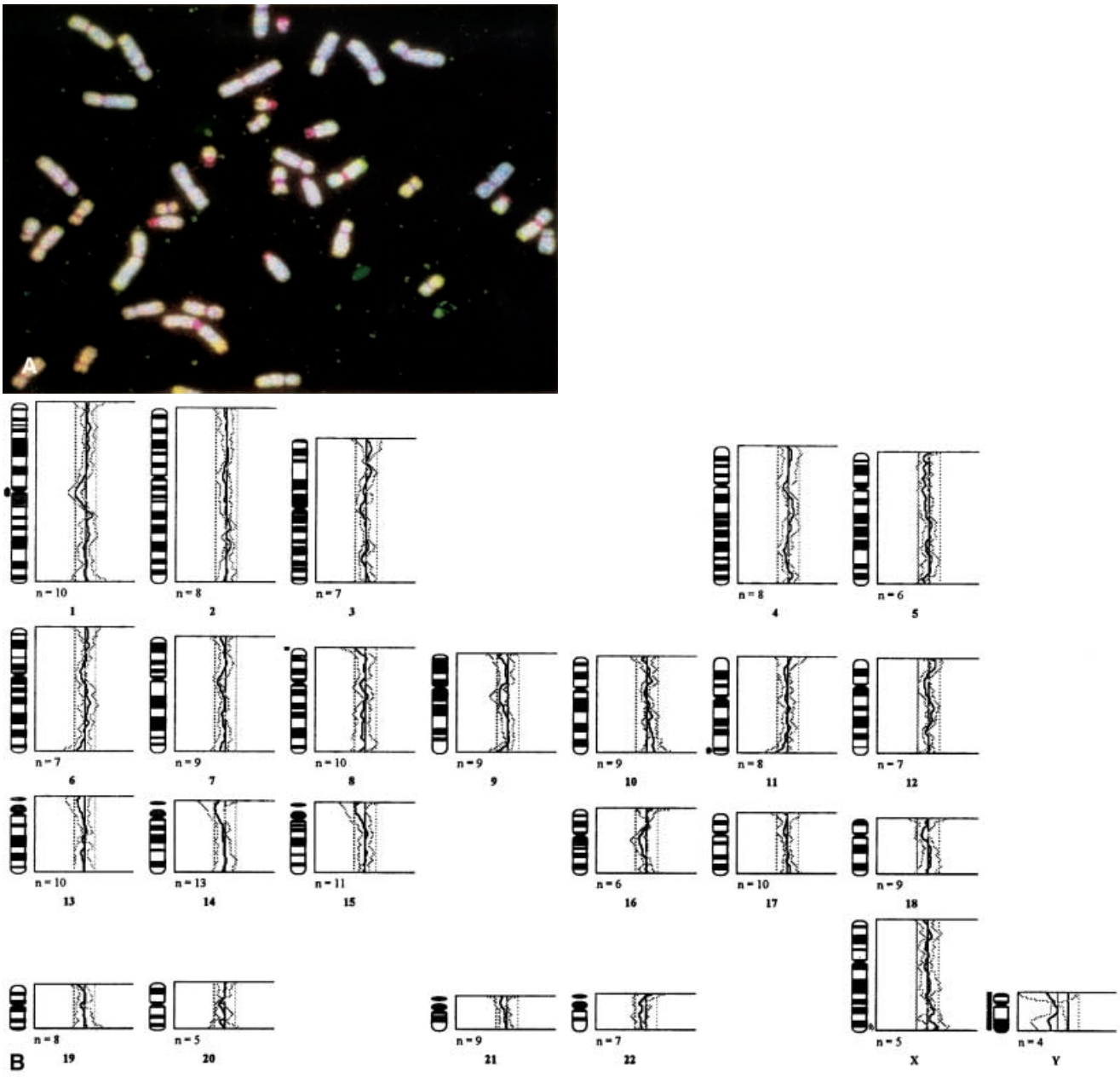

Fig. 5 **A** Comparative genomic hybridization (CGH) of DNA isolated from erythrocytes of patients with systemic lupus erythematosus (SLE) and hybridized on normal metaphases. Hybridization shows no prevalence of certain chromosomal regions, suggesting that the DNA bound to the erythrocyte surface is random, genomic DNA. The *red color* labeling of the telomere and centromere regions is due to repetitive sequences and is not evaluable. **B** Green to red ratio profile of the CGH with DNA from erythrocytes of SLE patients. For each chromosome, the mean±SD of the green to red ratio is shown. The *middle line* indicates a ratio of 1.0. The *dotted lines* indicate a ratio of 1.2 and 0.8

are cleaved and removed from circulation very efficiently. In SLE patients, these nucleic acids seem to persist and might eventually induce a B-cell-mediated immune response with the formation of anti-DNA antibodies [14, 33]. Here, we show evidence that a putative DNA-binding-like domain, which has receptor-like capabilities and is functionally impaired in SLE, might exist on the surface of erythrocytes. The DNA isolated from the surface of erythrocytes from SLE patients is of random chromosomal origin as demonstrated using CGH and seems to be tightly bound to the DNA-binding receptor of erythrocytes, while erythrocytes of healthy volunteer donors do not show any detectable amounts of nucleic acids. From our current knowledge about SLE, it is unlikely and has not been shown yet that there is significantly more cell death occurring in SLE. This DNA from apoptosis or necrosis is usually efficiently and quickly removed from the circulation. The DNA on the surface of SLE erythrocytes may also change the physical properties of red blood cells, probably reflected in a reduced cell electrophoretic mobility of SLE erythrocytes [13]. The persistent presence of DNA on the erythrocytes of SLE patients can also lead to the accumulation of cellular-bound

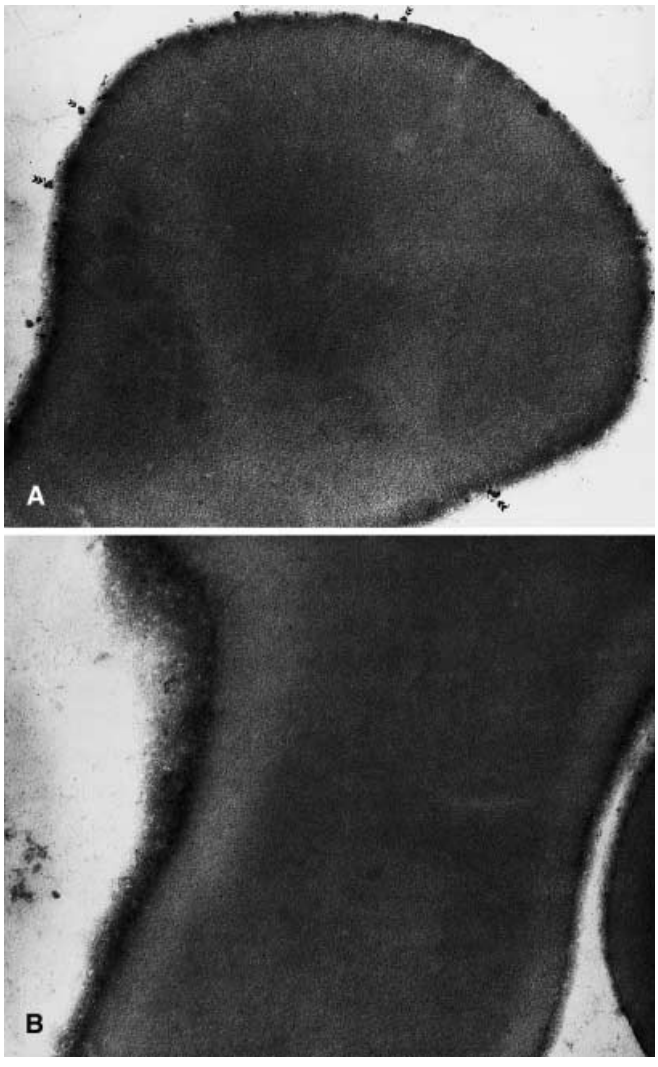

Fig. 6 Electron microscopy of erythrocytes from systemic lupus erythematosus (SLE) patients and normal controls. **A** Erythrocyte from an SLE patient (magnification ×30,000): the membrane surface shows electron-dense particles with an average size of 20–40 nm (*arrows*). This particle size correlates with DNA of 10 kb (Fig. 4). **B** Erythrocyte of a control person: the membrane surface only shows the glycocalyx but no larger electron-dense particles

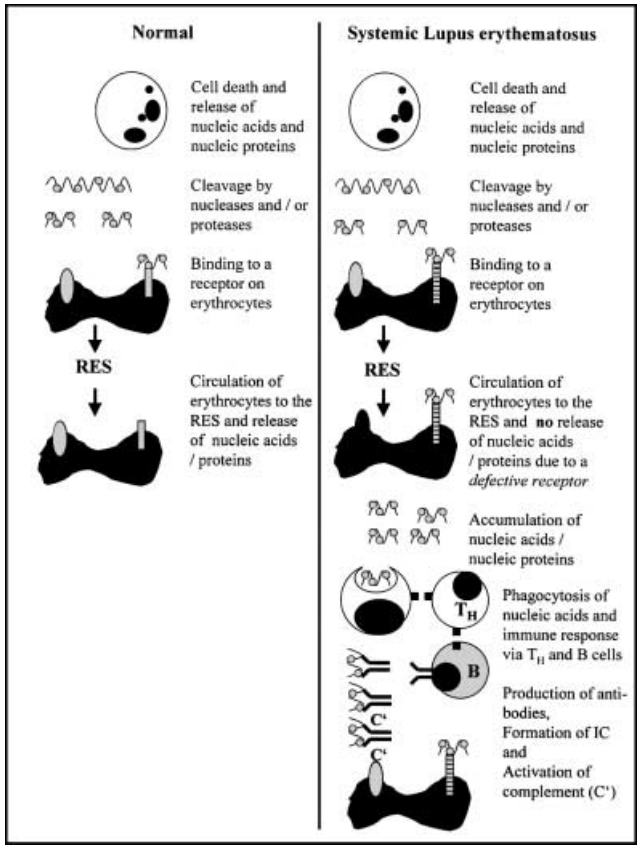

Fig. 7 Schematic presentation of the possible pathoetiology of systemic lupus erythematosus (SLE) due to a dysfunctional receptor on erythrocytes, leading to an accumulation of nucleic acids and their associated proteins, which induce a T_h- (T helper) and B-cell-mediated immune response. Nucleic acids bound to the erythrocytes of SLE patients saturate the DNA-binding receptor on SLE erythrocytes, while nucleic acids are usually presented to the reticulo-endothelial system (RES) and released from its binding domain. This binding function is independent of complement or antibodies

Table 2 Systemic lupus erythematosus (SLE)-promoting factors

Impaired reticuloendothelial system (mononuclear phagocyte system)
Reduced nucleases-activity
Immunogenetic prevalence (DR2 and DR3)
Complement deficiency (particularly C2 and C4)
Hormonal factors (female prevalence 9:1)

and free nucleic acids and might induce a B-cell-mediated immune response with the production of anti-DNA antibodies. These autoantibodies also recognize the DNA on the surface of erythrocytes, leading to opsonization and the activation of the macrophage system, which is directed against autologous erythrocytes. Macrophage/monocyte-mediated erythrophagocytosis is also observed in SLE.

Figure 7 summarizes the possible pathogenetic events in SLE based on our current findings, which could be explained by a defective DNA-binding receptor on the surface of SLE erythrocytes, which binds DNA irreversibly to the membrane surface and does not release DNA to the RES. Nevertheless, there are other predisposing factors that contribute to the clinical appearance of SLE (Table 2). However, these factors contribute either to a failure to eliminate nucleic acids from circulation using other clearance mechanisms [16] or represent an immune system which can be activated by the presence of released DNA [34]. The autoantibodies against DNA also show a reactivity with nucleic acid-associated proteins (Ro/SSA, Sm, RNP, cardiolipids, C1q, stress proteins) [5, 31], which can be explained by the release of nucleic acid–protein complexes of the complete genome. Rumore and Steinman analyzed circulating DNA in SLE bound as multimeric complexes to histone [27]. This his-

tone-bound oligosomal DNA is of small molecular size (200–800 bp) and is not consistent with the high-molecular weight DNA found on erythrocytes of SLE patients. Other phenomena, such as drug-induced SLE, are thought to be caused by hypo-methylation of DNA, which leads to overexpression of integrins and increased binding of T cells to macrophages and B cells [35].

As already postulated by others [2], we have evidence that a DNA-binding domain with receptor kinetics on the surface of erythrocytes actually exists and seems to be functionally impaired in SLE. The binding of DNA to this putative receptor is physiologically independent of complement or antibodies. Nevertheless, other mechanisms can compensate for the defective receptor on erythrocytes via complement activation and binding of immune complexes. However, they are unable to ablate the clinical progression of SLE. If the defective DNA-binding receptor on erythrocytes actually shows spontaneous or germ-line mutations, allogeneic hematopoietic stem cell or bone marrow transplantation could be a curative option, as already shown empirically [24]. In addition, one might speculate that an increased release of genomic nucleic acids into the periphery might lead to the saturation of the DNA-binding receptor on the surface of erythrocytes in SLE patients, which might at least contribute to the onset of the disease. A dysfunctional receptor on the surface of erythrocytes, however, would explain the persistent presence of DNA on erythrocytes, which can only be found in SLE patients.

Acknowledgements The authors thank Prof. Dr. B. Manger and Prof. Dr. B. Belohradzky for obtaining the blood samples and for their patient care. The electron microscopy was carried out at the Institute of Pathology, University of Erlangen-Nürnberg.

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