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Correlation of disease activity and Clq-binding immune complexes with the neutrophil inclusions which form in the presence of SLE sera

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SUMMARY

Intracytoplasmic inclusions containing immunoglobulin (Ig) and complement (C3) are found in normal neutrophils (PMN) after incubation with sera from patients with SLE. These inclusions are believed to be immune complexes removed by phagocytosis from the SLE patients' sera *in vitro*. Similar inclusions were also noted in the circulating PMN from some patients with SLE. In the present study we have examined the relationship between the presence of intracytoplasmic inclusions and various clinical and laboratory features of SLE. Blood from forty-five patients with SLE was drawn and separated at 37°C. Fresh heparinized blood was also obtained from normal volunteers and allowed to stand for 90 min at 37°C. The buffy coat cells from both normal and SLE groups were removed, centrifuged, washed and examined (direct method) or incubated in the SLE sera for 90 min at 37°C (indirect method). Slides of washed cells were prepared in the cytocentrifuge, stained with fluorescein-conjugated goat anti-human IgG, IgM, IgA and C3 and examined under ultra-violet light.

By the direct method, 24% of patients had small intracytoplasmic inclusions in their neutrophils when stained for IgG suggesting that immune complexes were phagocytosed *in vivo*. None of twenty-one normal controls had similar inclusions. By the indirect method, 62% of SLE patients were positive for IgG, 15% for IgM, 8% for IgA and 31% for C3. None of the twelve normal controls were positive.

By the indirect method, PMN inclusions containing both IgG and IgM correlated with clinical activity ($P<0{\cdot}001$), depressed serum complement (CH50, $P=0{\cdot}026$; and C3, $P<0{\cdot}051$), cryoglobulinaemia ($P=0{\cdot}014$), anti-nDNA antibodies ($P<0{\cdot}001$) and C1q-binding immune complexes ($P=0{\cdot}008$). A suggestive correlation with granulocytopenia was also observed. The presence of inclusions containing IgG alone did not correlate with any of these parameters. C3 and IgM appeared to be mutually exclusive, i.e. neither was present simultaneously. These findings suggest (1) that normal PMN on exposure to SLE sera develop intracytoplasmic inclusions by phagocytosis of immune complexes, (2) the presence of such complexes correlates with a number of parameters of disease activity, particularly when IgG and IgM are both present and (3) such complexes may be phagocytosed *in vivo* as suggested by the presence of inclusions *in vivo* and contribute to a number of granulocyte disturbances seen in patients with SLE. These abnormalities in granulocyte function may be important, predisposing factors for infection in patients with active SLE.

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INTRODUCTION

Phagocytosis of immunoglobulins and complement (C3) by normal neutrophils after incubation with sera from patients with systemic lupus erythematosus (SLE) has been reported (Cats, Lafeber & Klein, 1975; Steffelaar *et al.*, 1977). Such inclusions have also been noted by some investigators in the circulating neutrophils from patients with SLE (Steffelaar *et al.*, 1977; Vaughan *et al.*, 1968).

These neutrophil intracytoplasmic inclusions may have considerable clinical relevance, since SLE patients may have neutropenia (Michael *et al.*, 1951), neutrophil phagocytic defects (Brandt & Hedberg, 1969; Jasin, Orozco & Ziff, 1974), chemotactic defects (Landry, 1977) and inhibition of granulocyte colony-forming activity (Duckham *et al.*, 1975). Such defects may play a role in the increased risk of infection which is seen in patients with SLE (Dubois, 1966; Jessar, Lamont-Havers & Ragan, 1953; Ropes, 1964; Gerding *et al.*, 1970).

The presence of circulating immune complexes in the sera of SLE patients has been well documented. Such complexes may contain DNA and antibodies to DNA (Tan *et al.*, 1966; Robitaille & Tan, 1973; Arana & Seligmann, 1967; Koffler *et al.*, 1973). Their presence correlates with clinical activity (Bardana *et al.*, 1975; Schur & Sandson, 1968), decreased complement levels (Bardana *et al.*, 1975; Schur & Sandson, 1968; Pick, Levo & Weiss, 1974), presence of renal disease (Schur & Sandson, 1968; Pincus *et al.*, 1969) and vasculitis (Johnson, Edmonds & Holborow, 1973).

In the present study we have examined the relationship between the presence of intracytoplasmic inclusions and various clinical and laboratory features of SLE. It is suggested that such inclusions are comprised of immune complexes which may be phagocytized by neutrophils. Such phagocytosis may then contribute to impairment of function of the neutrophils of SLE patients.

MATERIALS AND METHODS

Patients. A total of forty-five patients with SLE were studied. All patients satisfied the criteria of the American Rheumatism Association (Cohen *et al.*, 1971) for classification of SLE. Blood for all tests was obtained on the same day. An activity score was devised as follows: one point each was given for fever, alopecia, arthritis, serositis, active cutaneous LE of recent onset, ESR > 50 mm/hr, leucopenia (WBC < 4,000 per mm^3), hypocomplementaemia, increased DNA binding and abnormal urinary sediment for a potential total of ten points if all were present.

Preparation of white blood cells for direct immunofluorescent staining: direct method. Fresh, heparinized blood was allowed to stand at 37°C for 60–90 min, the supernatant buffy coat cells removed, centrifuged at 150 *g* and washed three times in 0·9% normal saline. The buffy coat cells were then resuspended in 0·9% saline and the concentration adjusted to 4×10^6 cells in 1·5 ml saline. Using two to three drops per slide, the cells were then centrifuged in a Shandon cytocentrifuge (Shandon Instruments, Sweickley, Pennsylvania) onto glass slides pre-cleaned in a solution consisting of equal parts of 95% ethanol and 0·1 N HCl. Cells were then stained directly by the fluorescent antibody technique. This procedure was designated as the direct staining method.

Preparation of normal leucocytes incubated with patient sera: indirect method. Four million washed normal peripheral white blood cells obtained from healthy donors were suspended in 1·0 ml of fresh patient serum and incubated for 90 min at 37°C with gentle shaking. The cells were then centrifuged, washed three times in 0·9% saline and resuspended in 1·5 ml of saline. Slides of this suspension were then prepared for immunofluorescent staining by centrifugation in a Shandon cytocentrifuge. In addition, the patient's serum was also incubated with 4×10^6 of his own cells and prepared for staining as described above. Thus, both normal cells and patient cells were incubated in patient serum. This method was designated the indirect staining method.

Immunofluorescent staining of white blood cells. Slides of either patient cells or normal cells stained directly following separation (direct method) or normal cells incubated with patient serum (indirect method) were stained with fluorescein-conjugated goat antisera directed against human IgG, IgM, IgA and B_1C (Meloy Laboratories, Springfield, Virginia). The antibodies to the

immunoglobulins were shown to be mono-specific by immunodiffusion against purified immunoglobulins. Slides were examined with a Leitz–Ortholux microscope equipped with epi-illumination according to Ploem (1971) using an HBO 100 ultra high pressure mercury lamp with K490 and K510 fluorescence filters. Photomicrographs were taken with a Leitz Orthomat camera on high speed Ektachrome film (Eastman Kodak Co., Rochester, New York). Cells examined for the presence of cytoplasmic inclusions were graded as follows: 0 = no significant staining of neutrophils; trace = tiny to small inclusions in less than 50% of cells; 1+ = tiny to small inclusions in over 50% of cells; 2+ = small- to moderate-sized inclusions in less than 50% of cells, occasional cells containing large inclusions; 3+ = large inclusions seen in less than 50% of cells with the majority of cells containing smaller inclusions; 4+ = large inclusions in over 50% of cells. Control preparations in the direct method consisted of normal cells not previously incubated in serum. Control preparations in the indirect method consisted of normal cells incubated in the normal donor's own serum.

Parameters of disease activity. The following studies were carried out at the time of the inclusion studies: complete blood count and differential count, erythrocyte sedimentation rate (Westergren method), LE cell preparations and tests for cryoglobulins and the C3 component of complement. Antibodies to native DNA (nDNA) were measured by the method of Ginsburg & Keiser (1973). Total haemolytic complement, CH50, was measured by microtitration, using the method of Nelson *et al.*, (1966). Immune complexes were determined by the modified ^{125}I-C1q binding test, as described by Zubler *et al.* (1976).

Statistical calculations. The statistically significant differences between the various groups studied were calculated using χ^2 analysis.

RESULTS

Presence of inclusions in circulating neutrophils from SLE patients (direct method)

Utilizing the direct staining technique, 24% of the forty-five patients with SLE had small (1+ to 2+) neutrophil inclusions staining for IgG, 7% staining for IgM, 2% for IgA and 9% for C3. An

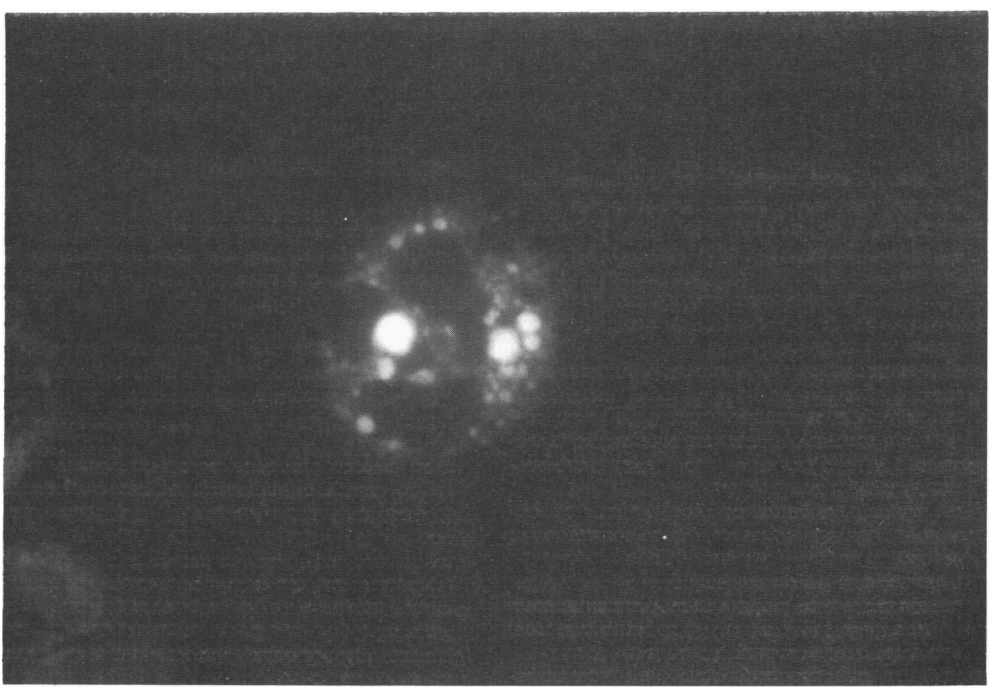

Fig. 1. Example of large intracytoplasmic inclusions which are present in some circulating neutrophils of SLE patients. In this preparation the inclusions are stained for IgG. (× 550.)

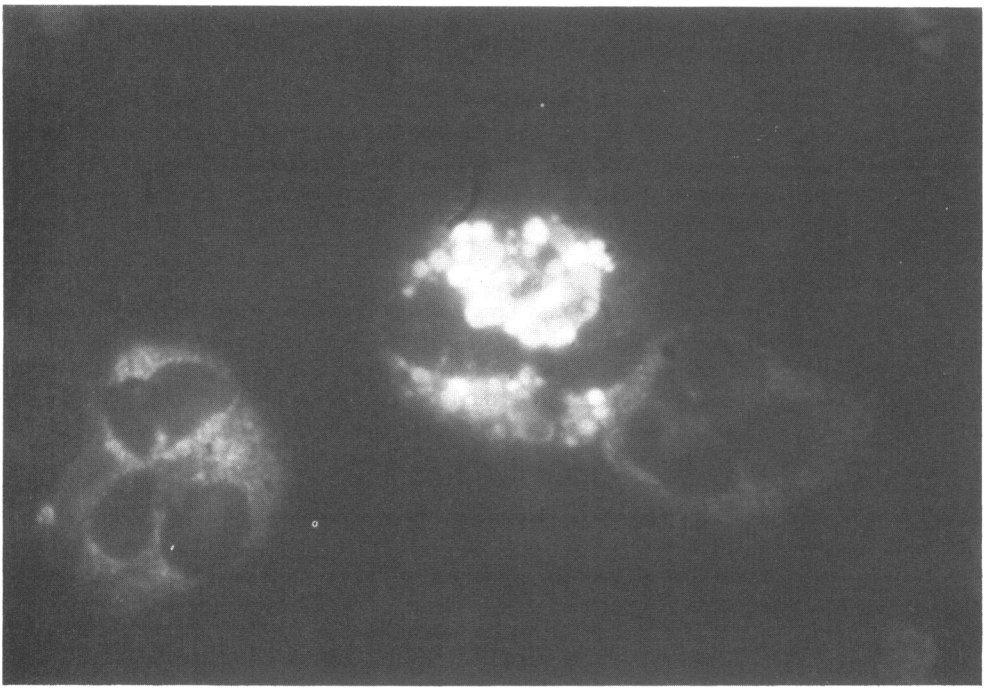

Fig. 2. Example of large intracytoplasmic inclusions which develop in normal neutrophils after incubation in SLE sera. In this preparation the inclusions are stained for IgG.

example of the type of inclusions detected with this technique is shown in Fig. 1. None of the twenty-one normal controls had any inclusions in their circulating neutrophils. While there was a suggestive correlation of the presence of inclusions with clinical activity, this was not statistically significant.

Correlation of indirect staining inclusions with various parameters of disease activity

An example of the type of intracytoplasmic inclusions which develop in normal neutrophils after incubation in SLE sera (indirect method) is shown in Fig. 2. Fig. 2 illustrates the type of large (3+ and 4+) intracytoplasmic inclusions seen using this method and in this instance is stained for IgG.

In contrast to the direct method, of the forty SLE patients studied by the indirect method, 62% were positive for IgG, 15% for IgM, 8% for IgA and 31% for C3. None of the twelve normal controls were positive.

The correlation of the presence of significant (3+ and 4+) indirect-staining inclusions with clinical activity is shown in Table 1. The inclusions were classified as either staining for IgG or both IgG and IgM. As mentioned previously, no significant IgA staining occurred. IgM staining alone occurred in only one instance.

As shown in Table 1, the IgG + IgM staining inclusions correlated with the highest mean clinical activity score (5·0). This average was significantly higher than that for IgG inclusions alone (mean 2·3; $P=0{\cdot}025$) or for no inclusions (mean 1·8; $P<0{\cdot}001$).

The correlation between the presence of inclusions and total neutrophil count was examined. Sera from six patients produced IgG + IgM staining inclusions. In these patients the mean number of total neutrophils per mm^3 was 3,375 (1,579–5,353). In the IgG-staining group, the mean neutrophil count was 5,006 per mm^3 (1,134–9,945). In the twenty sera which did not produce inclusions the neutrophil count was 6,204 per mm^3 (1,586–14,400). While the total neutrophil count was considerably lower in the IgG + IgM group, these values were not significantly different from the other two groups, presumably because of the small numbers in the IgG + IgM group.

When the correlation between different Ig classes of inclusions and LE cell tests were examined,

Table 1. Correlation of clinical activity score with cytoplasmic inclusions

Cytoplasmic inclusions	Number	Activity score (average)	*P* value	
IgG + IgM	7	5·0 (2–7)	0·025	
IgG	10	2·3 (0–6)		<0·001
Negative	18	1·8 (0–5)	n.s.	

n.s. = Not statistically significant.

Table 2. Correlation between inclusions and serum CH50 and C3 levels

Complement assay	Cytoplasmic inclusions	No. decreased/total no.	Per cent decreased	*P* value	
	IgG + IgM	6/8	75	n.s.	
CH50	IgG	7/14	50		0·026
	Negative	4/19	21	n.s.	
	IgG + IgM	5/5	100	<0·05	
C3	IgG	3/12	25		<0·05
	Negative	5/20	25	n.s.	

n.s. = Not statistically significant.

all three of the IgG–IgM inclusion-producing sera tested had positive LE cell preparations. Nine of thirteeen (68%) of the sera producing IgG-staining inclusions gave positive LE cell preparations and six of thirteen (46%) of the negative groups had positive LE cell preparations. This was not statistically significant. If the IgG–IgM group is combined with the IgG only group, then twelve of sixteen (75%) had positive LE cell preparations.

The correlation between presence of inclusions and serum complement levels is shown in Table 2. In the IgG–IgM group, six of eight or 75% had decreased CH50 levels as compared with seven of fourteen (50%) in the IgG group and four of nineteen (21%) in the negative group. The difference between the IgG–IgM group and the negative group was significant at the 0·026 level. The differences between the IgG–IgM and IgG groups and the IgG and negative groups were not statistically significant.

A decrease in serum C3 level also correlated with presence of IgG–IgM inclusions. As shown in Table 2, all five of the patients with IgG–IgM inclusions had decreased serum C3. This was significantly higher than either the IgG group (three of twelve or 25%, $P < 0{\cdot}05$), or the negative group (five of twenty or 25%, $P < 0{\cdot}05$).

The relationship of IgM-staining inclusions to C3-staining inclusions was also examined. Fifteen of thirty patients (50%) had neither C3 nor IgM staining, i.e. they were C3−, IgM−. Eight of thirty (27%) had C3−, IgM+. Seven of thirty or 23% had C3+, IgM−. Finally, none of the thirty patients had both C3+ and IgM+ inclusions. These data demonstrate that when IgM-staining inclusions were present C3 staining was never present. Conversely, if C3 staining was present, IgM staining was never present. Hence, C3 and IgM appeared to be mutually exclusive. Thus, IgG sometimes occurred with either IgM or C3 but never with both IgM and C3. IgG inclusions were

Table 3. Correlation between inclusions and presence of cryoglobulins

Cytoplasmic inclusions	No. positive/ total no,	Per cent positive	*P* value	
IgG+IgM	5/5	100	n.s.	0·014
IgG	5/13	38	n.s.	
Negative	4/16	25		

n.s. = Not statistically significant.

Table 4. Correlation between inclusions and anti-nDNA antibody levels

Cytoplasmic inclusions	No. positive/ total no.	Per cent positive	*P* value	
IgG+IgM	5/6	83	n.s.	<0·001
IgG	4/14	28	n.s.	
Negative	2/23	9		

n.s. = Not statistically significant.

also frequently present alone, without IgM or C3. As stated previously, no significant IgA inclusions were noted.

Cryoglobulins were present in the serum of all five with IgG–IgM inclusions that were tested (Table 3). Five of thirteen (38%) of the IgG group and four of sixteen (25%) of the negative group had serum cryoglobulins. The IgG–IgM group was significantly different compared to the group without inclusions ($P=0{\cdot}014$).

The correlation between inclusions and antibodies to native DNA (anti-nDNA) is shown in Table 4. Five of six (83%) of those with IgG–IgM inclusions had antibodies to nDNA in their sera, whereas only four of fourteen (28%) of the IgG group and two of twenty-three (9%) of the negative-inclusion group had anti-nDNA antibodies. The difference between the IgG–IgM and negative groups was statistically significant ($P<0{\cdot}001$).

The correlation between inclusions and the level of C1q-binding immune complexes is shown in Fig 3. It is apparent that as the inclusion score increases, the level of C1q-binding immune complexes also increases. This correlation was statistically significant ($r=0{\cdot}635$, $P=0{\cdot}008$).

DISCUSSION

These studies demonstrate a significant correlation between several parameters of disease activity and the development of intracytoplasmic inclusions which have presumably resulted from phagocytosis of immune complexes from SLE sera by normal neutrophils. Those parameters which were found to correlate with the presence in the same cell of both IgG- and IgM-staining inclusions include clinical activity, depressed serum complement (CH50 and C3 components), presence of cryoglobulins, anti-nDNA antibodies and C1q-binding immune complexes. A suggestive correlation with granulocytopenia was noted but this correlation was not statistically significant.

It is of interest that the best correlation occurred with those inclusions containing both IgG and

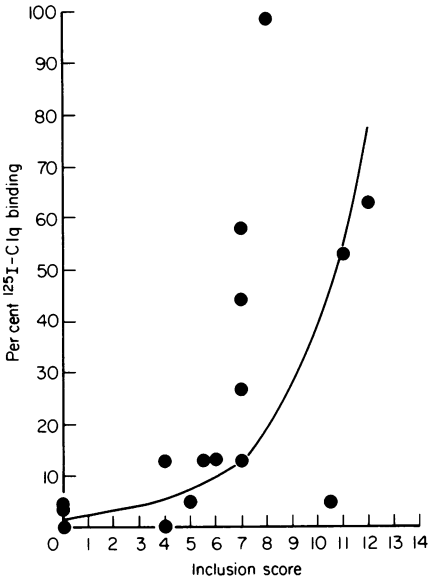

Fig. 3. Correlation of inclusion score with levels of ^{125}I-C1q-binding immune complexes ($r=0{\cdot}635$, $P=0{\cdot}008$). The inclusion score was derived by addition of numerical values for individual Igs and C3. Trace staining was assigned a value of 0·5.

IgM. This finding is similar to other studies which show that clinical activity and the magnitude of the DNA antibody response correlate with the number of Ig classes deposited beneath the epidermis (Sontheimer & Gilliam, 1979). While in some instances the presence of IgG-staining inclusions alone was associated with some of the above parameters, the coexistence of IgM and IgG appeared to correlate best with those indicators of more severe disease. This suggests that in the more active patient, a more vigorous antibody response is reflected by a larger number of different Ig classes.

It has been well documented that immune complexes containing nDNA and anti-nDNA are present in SLE patients and are pathogenetically related to glomerulonephritis (Schur & Sandson, 1968; Pincus *et al.*, 1969). The present studies strongly suggest that such immune complexes may be phagocytosed by the patients' own neutrophils or by neutrophils from normal donors. In studies to be reported elsewhere (Hurd & Gilliam, manuscript in preparation) using ethidium bromide which is known to stain double-stranded nucleic acids (Terman, 1961; LePecq, 1972; Paoletti, LePecq & Lehman, 1971), red fluorescence was noted in the intracytoplasmic inclusions in the patient's own cells and in inclusions which were phagocytosed from SLE sera suggesting that polynucleotides were present in such complexes. Such staining was not observed in control experiments in which inclusions were produced by incubating heat-aggregated IgG with normal neutrophils. In addition, when DNA was added to serum containing anti-DNA antibodies prior to incubation with normal neutrophils, the ethidium bromide staining increased in intensity, suggesting that anti-nDNA antibody was present in the inclusions, perhaps in antibody excess.

Concentration of DNA in the cryoprecipitates of patients with SLE has been demonstrated previously (Winfield, Koffler & Kunkel, 1975). SLE cryoprecipitates will also precipitate DNA (McPhaul, 1978). In the present studies the IgG–IgM inclusions correlated well with presence of cryoglobulins. Cryoglobulins present in SLE patients contain primarily IgG and IgM (Christian, Hatfield & Chase, 1963; Hanauer & Christian, 1967). It is not likely that the cryoglobulins *per se* were phagocytosed by the neutrophils since all experiments were performed at 37°C. It is likely, however, that the immune complexes which are phagocytosed at 37°C are soluble immune complexes which are the same as those which precipitate in the cold, i.e. cryoglobulins.

The finding that C3 and IgM staining in the inclusions were mutually exclusive suggests that the IgM in the immune complex may have rheumatoid factor activity. The IgM would then compete with complement for binding to IgG. That cryoprecipitates have immunological reactivity against

IgG has been reported previously (Brouet *et al.*, 1974; Grey & Kohler, 1973; Meltzer & Franklin, 1966).

The inclusions present in the patients' own cells tended to be much smaller and with a lower frequency than those produced *in vitro*. A possible explanation for the paucity of large inclusions noted in patient cells would be that such cells may be removed in one passage through the body, e.g. by the spleen or in other parts of the reticuloendothelial system. It has been shown that immune complexes are continually removed by the spleen after one passage through the circulation (Van Rooijen, 1975).

Granulocytopenia, as well as lymphopenia, may be seen in patients with SLE (Michael *et al.*, 1951). It is possible that increased margination or sequestration of neutrophils may occur following ingestion of immune complexes. In addition, impaired function may result from ingestion of immune complexes. A number of studies have demonstrated impaired neutrophil function (Taichman, Pryganski & Ranadive, 1972), increased release of lysosomal enzymes (Taichman *et al.*, 1972; Weissman *et al.*, 1971), decreased chemotaxis (Mowat & Baum, 1971) and impaired bacterial cell killing (Turner, Schumacher & Myers, 1973) after phagocytosis of immune complexes. The increased risk of infection seen in SLE patients (Dubois, 1966; Jessar *et al.*, 1953; Ropes, 1964; Gerding *et al.*, 1970) could possibly be related to such a granulocyte defect.

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