Thymus-Derived Rosette-Forming Cells in Various Human Disease States: Cancer, Lymphoma, Bacterial and Viral Infections, and Other Diseases

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ABSTRACT Lymphocytes that bind in vitro to sheep erythrocytes in a rosette formation are thymus-derived. A modified technique that does not detect the total number of rosette-forming cells (RFC) was used to study normal subjects and various disease states. Of 100 healthy subjects, 95 had more than 15% RFC (mean 28.4 ±6.5%). We studied 104 patients with solid tumors, who were classified according to clinical status and stage of therapy. Of 19 newly diagnosed patients, 13 had less than 15% RFC. Of 44 untreated patients undergoing relapse, 32 had less than 15% RFC. In both categories, patients with metastases had fewer RFC than patients with localized disease. 11 patients were studied 2 wk after cessation of therapy; four of them showed less than 15% RFC. Only one of 30 patients in remission had less than 15% RFC. In seven patients followed for various periods of time, the numbers of RFC correlated generally with clinical status. 11 patients with chronic lymphatic leukemia had very low percentages of RFC. 21 of 21 patients with symptoms of viral upper respiratory diseases had less than 15% RFC. RFC returned to normal values between 5 days and 7 wk after disappearance of clinical symptoms. 20 patients with bacterial infections had normal numbers of RFC. Of 25 patients with miscellaneous nonimmunologically related diseases, two had low numbers of RFC. It appears that the percentage of RFC may be valuable in evaluating not only immunological defenses but also the status of patients with solid tumors, lymphomas, viral diseases and, perhaps, bacterial infections.

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INTRODUCTION

Human peripheral blood lymphocytes will be surrounded in vitro by sheep erythrocytes (SRBC) [1] in a rosette formation (1–4). These rosette-forming cells (RFC) are thymus-derived cells (T cells) (5–11). In view of the importance of immune mechanisms in many diseases, it seems warranted to study the percentage of RFC in various clinical situations. Evaluation of RFC in patients with malignant diseases appears important for multiple reasons. It has been suggested, under the concept of immunosurveillance, that T cells and immune mechanisms can destroy an emerging clone of malignant cells (12–14). There is a close association between defects in cellular immunity and malignancies (15, 16). The general cellular immune mechanisms can be depressed in cancerous patients (17). Finally, it has been demonstrated that multiple specific immune mechanisms can be present in these patients (18). The present study indicates that patients with cancer have decreased percentages of RFC. Viral diseases also have been associated with decreased cellular immunity (19). This study reports low numbers of RFC in certain viral illnesses. Patients with bacterial infections, in contrast, have normal numbers of RFC. In chronic lymphatic leukemia, likely to be caused by clonal proliferation of B cells (20), low numbers of RFC are found. Various other diseases, not known to involve major immunological mechanisms, are not associated with decreased numbers of RFC.

[1] *Abbreviations used in this paper:* RFC, rosette-forming cell; SRBC, sheep erythrocytes.

METHODS

Subjects. We studied 100 normal subjects, 104 patients with solid tumors, 11 with chronic lymphatic leukemia, 20 with bacterial infections, 21 with viral diseases, 2 with mycotic diseases, and 31 with miscellaneous diseases. We excluded patients who had pure immunologic deficiencies.

The control group contained 44 males and 56 females with an age range of 14–89 yr (mean age, 44.7 yr). Three were Oriental, and the remainder were Caucasian. They denied any present or recent viral diseases or symptoms thereof. Six of the subjects had serial studies of RFC during a period of 9 mo. One of these volunteers contracted a viral disease (sore throat and rhinitis with negative bacterial cultures) during this period of observation.

108 solid tumors in 104 patients included 16 tumors of the breast, 15 of the colon, 13 of the lung, 7 of kidney, 5 of the pancreas, 4 of the prostate, 4 of the rectum, 2 of bone, 2 of the esophagus, 2 of the stomach, 2 of the thyroid, 1 each of the cervix, endometrium, gallbladder, nasopharynx, ovary, testicle, and vulva, 6 of unknown origin, 6 melanomas, 3 hemangiopericytomas, 2 rhabdomyosarcomas, 2 basal cell neoplasms, 1 clear cell neoplasm, and 1 squamous cell neoplasm. Four of these patients, whose tumors are not enumerated above, had histories of two malignancies: one had cancer of the breast and neurofibrosarcoma, one had cancers of breast and colon, one had cancers of the prostate and colon, and one had cancers of the bladder and rectum. These patients (58 women, 46 men) were between 18 and 82 yr of age (mean age, 47.3 yr). Three were Oriental; the remainder were Caucasian. They denied any present or recent viral diseases.

Patients with cancer were subdivided into six categories: 12 newly diagnosed patients with localized disease, 7 newly diagnosed with metastatic lesions, 14 with local relapse, 30 with metastatic relapse, 30 in remission, 7 with treated local recurrence, and 4 with treated metastatic disease. The treated patients included those who previously had had chemotherapy (5-fluorouracyl, cytoxan), radiation therapy, or both, but who had had no therapy during the 2 wk before study. All other patients with cancer (newly diagnosed, remission, relapse) had received no form of chemotherapy or radiation therapy for 2 yr before study; at least 1 yr had elapsed since the most recent surgery. A patient was considered to be in remission when he had remained free of disease for at least 1 yr after surgery or 2 yr after cessation of chemotherapy or radiation therapy.

Six patients with various neoplasms were followed for up to 6 mo. Four subjects were studied from one family with a high incidence of gastrointestinal carcinoma. The wife had 10 relatives in two generations who had had this type of cancer. We tested the wife, her husband, and their two children.

11 patients with chronic lymphatic leukemia (more than 30,000 lymphocytes/mm^3), two patients with newly diagnosed Sézary syndrome, and two with newly diagnosed generalized lymphosarcoma were also investigated. None of these 15 patients had received any therapy.

20 patients with bacterial infections (15 gram-positive, 5 gram-negative) were studied. There were 13 pulmonary infections (including three patients with lung abscesses), two pleural infections, three skin infections, and two urinary infections. Three of these patients had had repeated infections. All were receiving antibiotics. Two patients with mycotic diseases (mucormycosis and nocardia), who were also receiving appropriate antibiotics, were studied.

21 patients with clinically diagnosed viral diseases were studied. The diagnosis was based on common cold, rhinitis, flu-like syndrome, tracheitis, sore throat, or a combination of these symptoms. Some of the patients had mild leukopenia with or without lymphocytosis. Bacterial cultures, performed in some patients, were negative. Viral antibodies were not sought for. These diseases were self-limiting and did not require antibiotic therapy. Five of these patients were followed for various periods of time.

26 patients with miscellaneous diseases were studied: six with diabetes, four with heart failure, four with myocardial infarction, three with cirrhosis of the liver, and one each with Gaucher's disease, a phagocytic defect, mesenteric lipodystrophy, psoriasis, refractory anemia, renal failure, testicular cysts, testicular torsion, and valvular heart disease. All of these patients were newly diagnosed.

Five patients were studied who had diseases with some form of immunologic dysfunction: two had polyvasculitis, two had sarcoidosis, and one had scleroderma.

Methods. 10 ml of peripheral blood were drawn into a syringe containing 1,000 U of heparin (Lipo-Hepin, Riker Laboratories, Northridge, Calif.). Lymphocytes were isolated on a layer of Ficoll-Hypaque by one centrifugation at 400 *g* for 40 min (21). The ring, at the interface, contained approximately 90% lymphocytes and 10% monocytes. These cells were washed three times in phosphate-buffered saline (Dulbecco's formula; pH 7.4) and the final concentration was adjusted to 10^7 cells/ml; 0.05 ml of this suspension was added to each of five plastic tubes (13 × 100 mm, Falcon Plastics, Los Angeles, Calif.) containing 0.05 ml of gamma globulin-free calf serum, fetal calf serum, calf serum, bovine serum, or immunoprecipitated tested calf serum (Grand Island Biological Company, Berkeley, Calif.). All sera were heat-inactivated and absorbed against SRBC and human erythrocytes at 37°C and at 4°C. Cells from each subject were tested simultaneously with two to five sera.

The cells were incubated in a dry atmosphere of 95% air and 5% CO_2 for 1 h at 37°C. After this period, washed SRBC suspended in saline (final concentration 40×10^6/ml) were added to obtain a final ratio of eight SRBC to one mononuclear cell. The tubes were spun at room temperature for 5 min at 200 *g*; the pellet was gently suspended and a portion from each tube was placed in a hemocytometer with a Pasteur pipette. 200 or more lymphocytes from each were counted immediately under a light microscope at a magnification of 100 ×, and the percentage of lymphocytes that formed rosettes was calculated. An RFC was defined as a lymphocyte surrounded by at least three SRBC. For each subject studied, at least 1,000 cells were counted. The results represent the mean percentage of all simultaneous readings in each subject.

The study was done blindly, and the investigators did not know the origin of the samples.

RESULTS

The mean percentage of RFC in the 100 normal subjects was 28.4±6.5% (mean ±1 SD). Five subjects had values between 6 and 12%; the other 95 were between 16 and 41%. Therefore, the lower limit of normal has been defined as 15% RFC (mean of 100 normal subjects minus 2 SD). The five normal subjects who had less than 15% RFC had no apparent immunologic abnormalities or viral diseases. Results for five normal

TABLE I

Rosette Formation in Five Normal Subjects

Subject	Rosette-forming cells*
	%
A	25, 23, 26, 25, 27, 23
B	18, 20, 19, 21, 22, 18
C	30, 27, 30, 27, 31, 28
D	36, 36, 37, 33, 38
E	20, 19, 18, 21, 21

* Test was repeated at 2-mo intervals in each subject.

subjects followed for 9 mo are summarized in Table I. Variation in each subject was minimal during this period. One additional volunteer (subject F), who was followed serially, contracted a viral disease, and she is discussed below.

Patients with malignancies. Among patients with cancer, percentages of RFC differed according to their clinical categories (Table II). Patients with newly diagnosed localized lesions who had 15% or more RFC had melanoma or cancers of the kidney, esophagus, or rectum. The two newly diagnosed patients with metastatic lesions who had 15% or more RFC had carcinoma of the lung and carcinoma of unknown origin. Six patients with local relapse had 15% or more RFC; these patients had basal cell carcinoma, cancer of the lung, cancer of the pancreas, neurofibrosarcoma, cancer of bone, and cancer of unknown origin. Six patients undergoing metastatic relapse also had 15% or more RFC: two with cancer of the lung, and one each with cancer of the stomach, melanoma, hemangiopericytoma, and cancer of unknown origin. Only one patient in remission (breast cancer) had less than 15% RFC. The other patients in remission included 13 with cancer of the breast, 8 with cancer of the colon, 2 with cancer of the prostate, 2 with cancer of the rectum, 2 with cancer of the kidney, 1 with cancer of the testicle, and 1 with hemangiopericytoma. We observed, but have not yet fully investigated, that RFC of some patients, especially those with advanced lesions, have fewer SRBC surrounding the lymphocyte than do other subjects.

Among treated patients, four who had localized disease (two lung cancer, one thyroid, one esophagus) had more than 15% RFC, and three patients with metastatic disease (two colon and one lung cancer) had more than 15% RFC.

Study of the family with a history of gastrointestinal carcinoma indicated that the wife had 7% RFC, the son 11%, the daughter 32%, and the husband 35% RFC.

The 11 untreated patients with chronic lymphatic leukemia had 1–7% RFC. Two patients with Sézary syndrome had 7 and 9% RFC, and the two patients with generalized lymphosarcoma had 25 and 23%.

Follow-up of patients with malignancies. Seven patients were followed for various periods of time. One patient with osteosarcoma and without metastases was studied the first time 2 wk after radiation therapy and a first injection of transfer factor; she had 27% RFC. After each injection of transfer factor, her RFC showed a slight increase, e.g., from 26 to 33%. However, after 4 mo of continuous therapy (four injections of transfer factor) her RFC began to decline and reached zero 6 mo after the start of the study. At this time, she developed complete anergy in response to standard skin tests. During this time her tumor clinically and radio-

TABLE II

Rosette-Forming Cells in Normal Subjects and in Patients with Cancer

		Rosette-forming cells		Distribution of rosette-forming cells		
	n	Mean	SD	<15%	>15%	*P* value*
		%	%	*n*	*n*	
Normal subjects	100	28.4	±6.5	5	95	
Patients with cancer	(104)					
Newly diagnosed: local	12	12.2	±8.0‡	8	4	<0.00001
Newly diagnosed: metastases	7	8.1	±5.5‡	5	2	<0.00001
Local recurrence	14	14.0	±6.2‡	8	6	<0.00001
Metastatic recurrence	30	9.0	±5.4‡	24	6	<0.00001
Remission	30	23.7	±7.5	1	29	0.3787
Progressive, treated	7	20.1	±12.9	3	4	<0.01
Metastatic, treated	4	16.0	±11.6	1	3	0.19845

* Significance of the difference between the distribution of the control population and that of the various groups of patients with cancer.

‡ Significantly different from controls ($P < 0.01$).

logically remained unchanged. 2 mo after RFC began to decrease, her hematocrit began to drop, and 10 wk later one small metastasis appeared in the lung.

Another patient, who had received regional and general chemotherapy (methotrexate and 5-fluorouracil) for liver metastases of a breast malignancy during an 8 wk period before study, showed 11% RFC. During a period of 10 wk her RFC increased from 11 to 43% (intermediate value of 28, 52, 40%) associated with a dramatic clinical improvement and a normalization of her liver tests. During the period of study, she received low doses of prednisone (15 mg) and triiodothyroxine. Only 2 wk after the increase in her blood RFC, the cytotoxic property of her lymphocytes against breast tumor cells, previously absent, was demonstrable in vitro.

One patient with ovarian carcinoma had rapidly growing generalized metastases. Over a 3 wk period, her RFC decreased from 7 to 2%. One patient with a history of breast carcinoma had no residual lesion. Tested during a period of 6 mo, her RFC were stable at 18%.

One patient with hemangiopericytoma and local cutaneous relapse had 10% RFC. This local recurrence was resected. His RFC were 26 and 23% when retested 4 and 6 mo later. He is presently free of disease.

Two other patients were followed for 6 mo after removal of breast tumor and of tumor of the colon. They remained free of disease. Their numbers of RFC were stable, at 18 and 18%, and 16, 16, and 18%, respectively.

Patients with viral, bacterial, and mycotic infections. All patients with viral diseases had less than 15% RFC (Table III). Some patients were tested serially; their values returned to normal in a period varying from 5 days to 7 wk after remission of their symptoms (Table IV). One normal volunteer is also shown in Table IV. She contracted a viral disease and showed a marked drop in RFC with a return to previous values in about 6 wk; her clinical symptoms of flu-like syndrome and sore throat lasted only 3 wk.

One patient with carcinoma of the breast in remission presented with a flu-like syndrome with back pain. Extensive examination and tests excluded relapse of the carcinoma. Her blood RFC returned to normal values with the disappearance of her clinical symptoms.

In contrast, patients with bacterial infections always had more than 15% RFC. The two patients with mycoses also had more than 15% RFC.

Patients with miscellaneous diseases. Among the 26 patients with miscellaneous diseases, two patients (myocardial infarction, cirrhosis of the liver) were found to have less than 15% RFC. The two patients with sarcoidosis had 6 and 9% RFC. The three other patients with presumed immunologic dysfunctions (scleroderma and vasculitis) had 27, 34, and 31% RFC.

TABLE III

Distribution of Rosette-Forming Cells in Viral and Bacterial Diseases

	n	<15%	>15%
Viral diseases	21	21	0
Bacterial diseases	20	0	20

DISCUSSION

The peripheral blood lymphocytes that form rosettes in vitro with sheep erythrocytes are thymus-derived cells (T cells) (5–11). Our previous studies indicate that in human fetuses between 11 and 19 wk of gestational age, the majority of RFC are found in the thymus and not in other organs (5, 6). On the basis of these findings, we proposed that RFC in adult blood are T cells. The technique used, however, produced low normal values and wide variation in values for the same individual tested serially.

The present technique, termed the "active rosette test," eliminates most of these disadvantages while retaining the ability to evaluate T cells. Indeed, with this technique we have shown that fetal rosette formation in the presence of serum increases in the thymus, blood, spleen, and liver (11). In contrast, rosette formation in the fetal bone marrow is abolished under these conditions. Thus, serum affects rosette formation in the same way in the fetal thymus and in the blood of fetuses and adults.

Up to 90% of fetal thymocytes are RFC around the 15th–16th wk of gestation; thereafter, the numbers of

TABLE IV

Variations in Rosette-Forming Cells in Patients with Viral Diseases

Subject	Rosette-forming cells		
	Before	During	After
	%	%	%
F*	26, 25	5, 8	16, 13, 17, 19, 25, 27
G		12	13, 27, 29
H		10	31
I		11	14, 20, 21, 20
K		8	20
L		3	11, 24, 26

* In this subject, a normal volunteer, RFC returned to previous values in 7 wk; subjects G and I had low values for 2 wk even though the clinical symptoms lasted only 5 days. Subject K is a cancer patient in remission who had flu-like symptoms associated with decreased rosette-forming cells.

thymic RFC decrease. Apparently, not all thymocytes form rosettes with SRBC, suggesting that rosette formation under these conditions identifies only certain subpopulations of T cells. At the same time the number of fetal RFC begins to decrease, the cortical area of the thymus enlarges (22). This may suggest that RFC belong to the medulla of this organ. In mice, the medulla is postulated to contain the functional immunocompetent T cells whereas the cortex has nonfunctional T cells (23). If this were also true in humans, it would imply that RFC, as detected by this technique, represent the population(s) of T cells involved in the active aspects of cellular immunity. Other techniques, which detect more RFC, may measure the total number of T cells (10). Although it is difficult to demonstrate that RFC, as detected in the present study, are in fact, functional, this concept would certainly be consistent with the data obtained in patients with immunodeficiencies and with cancer.

The reproducibility over time of the technique is demonstrated by the fact that serial studies of normal volunteers showed only minimal variations in numbers of blood RFC (Table I). The assignment of 15% RFC as the lower limit of normal allows us to detect more easily abnormalities which are likely to reflect a decreased number of T cells or a decreased cellular immunity. All of the patients with known defects in cellular immunity (Nezelof syndrome, Wiskott-Aldrich syndrome) have decreased RFC; among patients with Wiskott-Aldrich syndrome, only those who respond to transfer factor by clinical and immunologic criteria will significantly increase their blood RFC (24).

In the present study, two patients with sarcoidosis and documented anergy had low numbers of RFC. The 11 patients with chronic lymphatic leukemia had also low numbers of RFC. The latter finding was to be expected because this disease is thought to represent a proliferation of B cells (20). Three patients with vasculitis had normal or high numbers of RFC. These last results are intriguing, especially since the cellular immune system has not been extensively investigated in such diseases.

Patients with miscellaneous nonimmunologically related diseases had RFC in the normal range, despite the fact that many were extremely ill. It thus appears that debilitation is not a factor causing decreased RFC in the active test. The groups of patients with bacterial infections is interesting. Here, too, many patients were debilitated and, therefore, provide a good control group for the patients with cancer who had advanced disease. All patients with bacterial infections had normal numbers of RFC. The number of patients studied is too limited to conclude that the test may be helpful in the differential diagnosis of bacterial infections from other diseases. In this respect, however, all patients with probable viral diseases showed low numbers of RFC. Where follow-up could be performed, RFC returned to normal values between 5 days and 7 wk after cessation of clinical manifestations. Here, too, the small number of patients studied and the fact that only certain diseases (common cold, flu-like syndrome, sore throat) were studied, makes premature any conclusions about the usefulness of this test in all viral diseases. Nevertheless, if future data confirm that viral diseases are associated with low numbers of RFC, this test would be helpful in the diagnosis of such diseases.

The mechanism(s) by which RFC are depressed in this group of patients have not been investigated, but several hypotheses can be offered. Viruses may sterically hinder the receptors for SRBC, or may turn off the synthesis of such receptors. Viruses may alter the lymphocyte metabolism so that modifications of the lymphocyte membrane prevent rosette formation with SRBC. Viral diseases may increase the number of B cells. Finally, certain viral diseases are known to be associated with defects in cellular immunity, which may reflect a decrease in the number of functional T cells (19). No other immunological evaluation was performed in our patients; therefore, all these speculations remain hypothetical.

Some striking points emerge when the patients with cancer are grouped according to clinical status without regard for type of cancer. Of 30 patients in complete remission receiving no therapy, only one had less than 15% RFC. All the others were above this value, and their mean value was not different from that of the control population. These results are in sharp contrast with those observed in the other untreated patients. Only 30% of newly diagnosed patients with localized disease and 28% with metastatic disease had more than 15% RFC. Similarly, a significant number of patients in relapse had low numbers of RFC, whether their tumor was localized or had metastatized. Thus, it appears that the presence of tumor, even if very localized (in some cases, biopsies indicated that the lesion was restricted to the mucosa), can be associated with a decreased number of RFC. Whether the presence of the tumor produces the drop in RFC or the low number of RFC precedes the appearance of the tumor remains speculative. Only prospective studies can answer this question. In one case of osteosarcoma, a drop of RFC preceded by several weeks the appearance of a lung metastasis.

The number of patients studied during therapy was small. It appears, however, that values above 15% are not uncommon (7 of 11 compared with 18 of 63 among untreated patients). It is known that response to therapy may be associated with increased cellular immunity

(25–27). In the present study, although no follow-up of RFC is yet available in treated patients and no clinical correlation is possible, it is very tempting to relate the high number of patients with normal RFC to the above observations. In a study that looked at total RFC in patients after presternal irradiation for mammary carcinoma, the numbers of RFC were decreased for at least 1 yr (28). The response to phytohemagglutinin was also reduced. Our findings with the active rosette test, which does not detect all T cells, appear to contradict these earlier results. However, techniques, choice of patients, and type of therapy differed so that comparisons between the two studies are difficult. Furthermore, only serial studies will enable us to determine if RFC, as measured by the active test, increase during therapy. The mechanism proposed to explain an "overshoot" in cellular immunity (selective destruction of lymphocytes, release of immunogenic material during therapy) could explain an increase in RFC.

Can a defect in cellular immunity, as postulated in the concept of immunosurveillance, precede the appearance of tumors? The rosette system seems to be a tool to investigate this problem. Indeed, if the emergence of a clone of malignant cells signifies the absence or nonfunctional status of the population(s) involved in tumor killing, one would expect that a decrease in RFC will precede the manifestations of tumor. It is suggestive that, in a high-risk cancer family, two relatives have low numbers of RFC. As stated in the results, 5% of the controls have less than 15%. Therefore, we plan active follow-up of the subjects from high-risk families and of the normal control subjects who had low numbers of RFC, since a low number of RFC may indicate a predisposition to disease. In this regard, we have seen another patient, not reported in detail here, who was hospitalized for immune thrombocytopenia. He was receiving 100 mg of prednisone daily at the time of study; his RFC were 0%. 4 mo later, he died of myocardial infarction; a small malignant tumor was found in one kidney. This case has, however, many factors that might account for reduced cellular immunity (autoimmune disease, steroids), and it is difficult to draw any conclusions.

The numbers of RFC appear to correlate closely with the clinical status of the patients with cancer that we studied. It appears that absence of tumor is associated with a stable number of RFC. In one patient, an increase of RFC to very high levels was associated with a return to normal liver function. This increase is likely to reflect an increase in cellular immunity. Indeed, her lymphocytes became cytotoxic against breast tumor cells 2 wk after the increase of RFC. This boost in cellular immunity may be related to therapy. Indeed, as mentioned above, it has been shown that the response of lymphocytes to phytohemagglutinin increases after successful chemotherapy. In a patient with osteosarcoma, a drop in RFC occurred 6 wk before any other laboratory sign of deterioration (drop in hematocrit) and 10 wk before appearance of a metastatic lesion in one lung.

In conclusion, patients with cellular immune defects, chronic lymphatic leukemia, viral diseases, and growing neoplasms generally have decreased numbers of RFC. In patients with cancer, there appears to be an association between clinical status and number of RFC. That this association is not always demonstrable is to be expected in view of the numerous immunologic factors other than cellular immunity that are operative in these patients. It is not at all unlikely that, once a tumor grows, it stimulates the immune mechanisms of the patient and increases his number of active T cells. Whether RFC are part of the direct cellular immune defense against cancer or whether they reflect other mechanisms of defense remains to be demonstrated.

Note added in proof. Since submission of this manuscript, we have used a method that detects the total number of RFC (29). We have found that the majority of patients with malignancies have normal percentages of total RFC. Patients with metastatic disease can, however, have low percentages of RFC. We have also isolated from peripheral blood of patients with malignant solid tumors a T cell-rich fraction, and have found that these cells can specifically kill tumor cells in vitro, whereas the T cell-poor fraction from the same donors, containing B cells, monocytes, and few T cells, has decreased or absent killing activity (30).

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