FUNCTIONAL CHARACTERIZATION OF A STABLE, NONCYTOLYTIC STAGE OF MACROPHAGE ACTIVATION IN TUMORS*

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Stimulation either in vivo or in vitro by any of a number of biological or chemical agents (1–3) will render macrophages (MΦ)[1] nonspecifically cytolytic for tumor target cells in vitro. MΦ which have acquired such lytic activity have often been referred to as activated. By this operational definition, MΦ recovered from certain neoplasms, for example regressing Moloney sarcomas (4), have been activated in vivo, as they will kill a wide variety of antigenically distinct tumor target cells when freshly explanted into culture. MΦ isolated from many tumors have proved to be inefficient killers, however, or have lacked cytolytic activity entirely (5–7). This can be the case even within the Moloney system as MΦ obtained from progressing, rather than regressing, sarcomas usually lack the ability to destroy tumor cells (8). In view of the importance that cytolytic MΦ within tumors could have in host defense against neoplasia, the question of why MΦ from some tumors will kill, while those from the majority will not, becomes an urgent one. The data to be presented here we believe are the first that directly begin to resolve this enigma.

Mouse peritoneal MΦ elicited by the injection of thioglycollate broth (TG-MΦ), will become cytotoxic for tumor target cells when they are exposed to microgram/milliliter concentrations of bacterial lipopolysaccharide (LPS, endotoxin) in vitro (3).[2] LPS concentrations in the microgram/milliliter range also have been shown to induce cytotoxicity in MΦ recovered directly from mouse or rat fibrosarcomas (5). Thus, under appropriate conditions LPS is able to activate MΦ from a variety of sources. We will show, however, that a distinct

[1] *Abbreviations used in this paper:* FBS, fetal bovine serum; H-MEM, Eagle's minimum essential tissue culture medium containing Earle's salts and buffered with 15 mM HEPES; LPS, bacterial lipopolysaccharide; MΦ, macrophage; MSC, Moloney sarcoma of strain BALB/c origin; regressor or progressor MΦ, a MΦ recovered from either a regressing or progressing neoplasm, respectively; SV40-3T3, BALB/c fibroblasts transformed by SV40 virus; TG-MΦ, macrophage elicited from the peritoneal cavity by the intraperitoneal injection of thioglycollate broth.

[2] W. F. Doe, and P. M. Henson. Macrophage stimulation by bacterial lipopolysaccharides. I. Cytolytic effect on tumor target cells. Manuscript submitted for publication.

difference in LPS-responsiveness exists between peritoneal and tumor MΦ, the latter being much more sensitive to stimulation. Evidence that such heightened responsiveness is indicative of an important functional change which develops in tumor MΦ, and that this change enables these primed cells to respond to a final signal or combination of signals by becoming cytolytic, will be provided. Finally, we will show that MΦ in progressing sarcomas, at least those studied here, are noncytolytic because they have been activated only partially, rather than fully, and not because there is a total absence of MΦ stimulation in neoplasms.

Materials and Methods

Tumor Systems. The mice used were 6- to 10-wk-old BALB/c AnCr males. Tumors were induced by intramuscular (gastrocnemius) injection of either cultured MSC (Moloney sarcoma of BALB/c origin) cells (9) or 3T3 cells transformed by SV40 virus (10) contained in 20 μl of HEPES-buffered (15 mM) Eagle's minimum essential tissue culture medium (H-MEM). As shown previously (11), most mice injected with 5×10^3 MSC cells develop tumors which regress spontaneously, while inoculation of 10^6 MSC cells causes neoplasms which are invariably progressive and lethal. All tumorigenic doses of SV40-3T3 cells (here 10^5) produce neoplasms which grow inexorably. For these experiments sarcomas were harvested aseptically either 11 (regressing) or 14 (progressing) days postinoculation, except where indicated otherwise.

Tumor Disaggregation. As described in detail elsewhere (12), tumors were minced and disaggregated by repeated exposure to a stirred mixture of trypsin (2.5% solution, Grand Island Biological Co., Grand Island, N.Y.), crude collagenase (CLS II, 179 U/mg, Worthington Biochemical Corp., Freehold, N.J.), and deoxyribonuclease (DNAse I, B grade, 1.2×10^5 dornase U/mg, Calbiochem, San Diego, Calif.). The former two enzymes were used at concentrations of 0.1 mg/ml, and the DNAse at 0.025 mg/ml. After discarding the first two harvests (which eliminated most dead cells and debris), cells freed from fragments were centrifuged out of the enzyme solution at 20-min intervals and held pooled in ice-cold H-MEM containing 10% fetal bovine serum (FBS, Flow Laboratories, Inc., Rockville, Md.).

Cytotoxicity Assay. MΦ in cell suspensions derived from tumors were established in monolayers, as reported previously (4). Briefly, 2.5×10^6 cells in 0.5 ml H-MEM + 10% FBS were seeded into each of the 16-mm diameter, flat-bottomed wells of plastic LINBRO plates (Flow Laboratories, Inc.). After 5 min the plates were vigorously shaken to resuspend nonadherent cells, after which another 5 min of incubation was allowed. Plates were again shaken after a total elapsed time of 10 min and the supernatant medium containing nonadherent cells was aspirated. The entire procedure was repeated twice more. After the third plating, between 0.6 and 1×10^6 cells usually remained adherent to the plastic. This determination was made by direct counting of methanol-fixed, Giemsa-stained cells in representative wells. A microscope ($\times$ 400 magnification) equipped with an ocular grid of known area was used for counting. Variability in total cell number well-to-well was <10%. MΦ population density (MΦ/mm^2) is used throughout this paper to characterize monolayers, rather than the total number of MΦ/well or the ratio of MΦ to target cells, because the density of MΦ in monolayers has been shown to be one of the most important culture variables influencing killing.[2] The percentage of MΦ in monolayers was determined by counting the total number of phagocytic cells in wells to which zymosan had been added before fixation, and correcting for polymorphonuclear leukocytes. Effector cell populations prepared from Moloney sarcomas always contained >80% MΦ, and usually >90%, while those from SV40-3T3 sarcomas were >70%. The most common contaminating cell types, which previously have been shown not to interfere with the assay of MΦ-mediated cytotoxicity (4), were, in order of frequency, tumor cells, polymorphonuclear leukocytes, and rare T lymphocytes.

As before (4), release of ^{51}Cr from prelabeled P815 mastocytoma cells was used as the measure of MΦ-mediated killing. These targets are antigenically unrelated to the MSC or SV40-3T3 cells used to induce tumors and therefore were insusceptible to specific killing by the rare T lymphocyte which contaminated monolayers, (13). Monolayers were examined microscopically at the end of the ^{51}Cr-release assay (16 h) and again 48 h later to estimate the number of target cells

remaining. The results of these visual evaluations correlated inversely with the percentage of ^{51}Cr released during the assay period. Assay wells were set up in triplicate for each sample as follows: 0.5 ml of H-MEM + 60% FBS, then 0.5 ml of H-MEM with or without an appropriate concentration of LPS, followed by 0.5 ml H-MEM + 30% FBS containing 10^5 prelabeled target cells. The final FBS concentration in a total vol of 1.5 ml was 30%. After 16 h, the contents of each well were mixed by drawing 1 ml of the total volume up and down once in an automatic pipette (Selectapette, Clay Adams, Inc. Div. of Becton, Dickinson & Co., Parsippany, N.J.), after which 1 ml was removed and centrifuged to eliminate cells. A 0.5-ml aliquot of centrifuged supernate was removed from these samples for counting in a gamma scintillation spectrometer. Results are reported as percent specific ^{51}Cr release, calculated as follows:

$$\frac{\text{Experimental release} - \text{spontaneous release}}{\text{Total release (freeze-thaw 3 times)} - \text{spontaneous release}} \times 100$$

Spontaneous release of ^{51}Cr (1.5–2% per hour) was that which occurred from prelabeled P815 mastocytoma cells incubated during the assay period on monolayers of TG-MΦ.

Bacterial LPS. Purified LPS for these experiments was provided by Dr. David C. Morrison (Scripps Clinic and Research Foundation). Preparation of this phenol-extracted, lipid A-rich molecule (fraction II) from *Escherichia coli* serotype 0111:B4 (ATCC 12015) has been described (14). Quantification of LPS was accomplished by using a modification (14) of the colitose assay first described by Cynkin and Ashwell (15).

Results

Effect of LPS on MΦ Isolated from Regressing Moloney Sarcomas. The TG-MΦ of BALB/c mice are relatively refractory to in vitro activation by LPS; at least levels of killing mediated by these cells are lower than those which can be attained by using the TG-MΦ of many other strains, for example, C57BL/6.[3] We therefore wanted to ascertain first whether or not the MΦ isolated from tumors of BALB/c mice were similarly refractory. MΦ monolayers were established from 11-day-old regressing Moloney sarcomas and immediately challenged with target cells, with or without added LPS (10 μg/ml). ^{51}Cr release in unstimulated cultures was 41%, while that obtained from LPS-stimulated monolayers was 84%. Visual examination of the cultures confirmed that the greatest degree of killing had occurred in the wells which contained both tumor MΦ and LPS. In a series of nine experiments conducted under similar conditions, TG-MΦ obtained from normal BALB/c mice gave a mean ($\pm$ standard deviation) ^{51}Cr release of 11 $\pm$ 8%. Based on these data it was concluded that, unlike MΦ obtained from thioglycollate-stimulated peritoneal cavities, MΦ isolated from regressing Moloney sarcomas of BALB/c mice were highly responsive to stimulation by LPS.

To determine the smallest amount of LPS that would enhance killing by regressor MΦ, and thus their degree of LPS-sensitivity, a dose-response assay was performed over a concentration range of 10 fg to 10 μg/ml. Fig. 1a illustrates that at a MΦ population density of 6 × 10³ MΦ/mm² LPS concentrations $\geq$1 ng/ml gave maximal stimulation, with the linear portion of the dose-response curve falling in the picogram/milliliter range. By comparison, TG-MΦ similarly exposed to LPS failed to kill, except to a slight degree at an LPS concentration of 10 μg/ml.

Effect of LPS on Regressor MΦ Which Had Lost Cytolytic Activity In
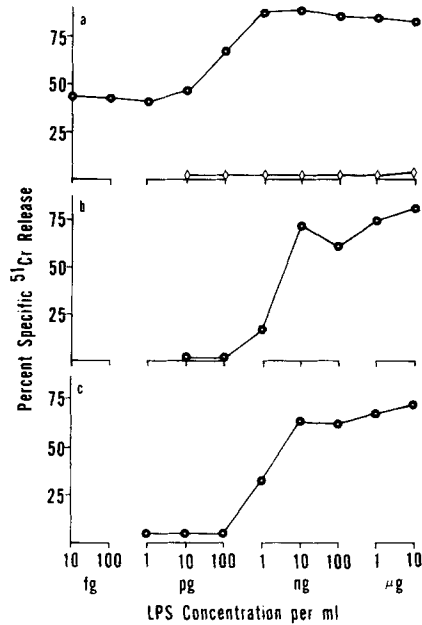

FIG. 1. MΦ from an 11-day, regressing Moloney sarcoma were seeded into wells and either challenged immediately with 10^5 P815 mastocytoma target cells in the absence or presence of various concentrations of LPS, or they were held in culture for 24 or 72 h before cytotoxicity was assayed. 1 a. Immediate challenge. Effect of increasing concentrations of LPS added either to monolayers consisting of tumor MΦ (O—O) or TG-MΦ obtained from normal mice (◇—◇). Only tumor MΦ responded, the linear portion of the LPS dose-response curve being found in the picogram to nanogram/milliliter range. Without added LPS, tumor MΦ effected 46%-specific ^{51}Cr release, while 0% was obtained by using TG-MΦ. 1 b. After 24 h in culture. Capacity of the tumor MΦ to kill without additional stimulation had been lost: ^{51}Cr release was 0% at LPS concentrations ≤ 100 pg/ml. LPS sensitivity was pronounced. 1 c. After 72 h in culture, with supernatant medium having been replaced after 48 h. ^{51}Cr release was 5% without LPS, or at concentrations ≤ 100 pg/ml. A similar result was obtained in a separate experiment after tumor MΦ had been held in culture for 96 h (two medium changes) before target cells and LPS were added.

Vitro. MΦ derived from regressing sarcomas lose their capacity to kill tumor cells after 24–48 h in culture (4). To determine whether such formerly cytotoxic MΦ remain sensitive to minute amounts of LPS or, functionally became more like poorly responsive TG-MΦ, monolayers were established from 11-day regressing Moloney sarcomas and cultured for various times before they were exposed to LPS and challenged with target cells. Monolayers were insignificantly cytolytic after 24 and 72 h in vitro, unless they were stimulated by LPS (Figs. 1 b and 1 c). The concentration which induced maximal cytolytic activity was 10 ng/ml, slightly more than the amount that was needed to induce freshly explanted regressor MΦ to kill at peak levels (Fig. 1 a). It is difficult to ascribe significance to this difference, however, because monolayers had diminished in MΦ population density (from 6×10^3 to approximately $4 \times 10^3/\text{mm}^2$) during the periods of incubation in vitro. In a separate experiment (data not shown), such exquisite sensitivity to LPS remained a characteristic of regressor MΦ held up to 96 h in culture.
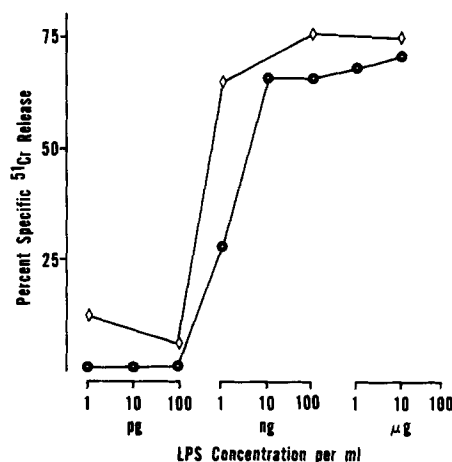
FIG. 2. LPS-stimulated killing by MΦ recovered from progessing Moloney (O—O) and SV40-3T3 (◇—◇) sarcomas. In both instances LPS concentrations of approximately 1 ng/ml induced maximal killing from the otherwise impotent progressor MΦ.

Effect of LPS on MΦ Recovered from Progressing Sarcomas. Even when challenged with target cells immediately after explantation, MΦ from large, progressing Moloney sarcomas are inefficient killers, compared with those isolated from regressing sarcomas (8). MΦ recovered from progressing sarcomas induced by SV40-3T3 cells are similarly impaired in their ability to kill in vitro (S. W. Russell, unpublished data). To determine their sensitivity to LPS, MΦ from each of these progressing sarcomas (both postinoculation day 14) were established in vitro and separately challenged with tumor target cells, either in the absence or presence of various concentrations of LPS. As shown in Fig. 2, these progressor MΦ developed the capacity to kill tumor target cells when they were exposed to the same concentrations of LPS that were effective in amplifying the cytolytic activity of MΦ derived from regressing Moloney sarcomas.

Is Heightened Responsiveness to LPS a Systemic Phenomenon in Tumor-Bearing Mice? To determine whether MΦ throughout the bodies of tumored mice, or only those within the milieu of the neoplasm, were characterized by heightened LPS responsiveness we undertook a study of TG-MΦ elicited from mice bearing either regressing or progressing Moloney sarcomas. The LPS responsiveness of these cells was compared either to TG-MΦ obtained from normal mice or MΦ recovered directly from regressing Moloney sarcomas. Fig. 3 shows that, in contrast to MΦ derived from tumors, LPS at concentrations up to 10 μg/ml failed to induce significant cytolytic activity in any of the TG-MΦ populations. The presence of a tumor elsewhere in the body therefore did not alter the pattern of LPS-responsiveness of peritoneal MΦ.

Kinetics of ^{51}Cr *Release from Target Cells Injured by LPS-Stimulated MΦ.* Sequential determinations were made of ^{51}Cr released from target cells by either LPS-stimulated (100 ng/ml) or unstimulated MΦ freshly isolated from regressing Moloney sarcomas. The objective was to determine whether the release profiles for the two effector cell populations were basically the same or different. Fig. 4 shows that they were similar. In each instance specific
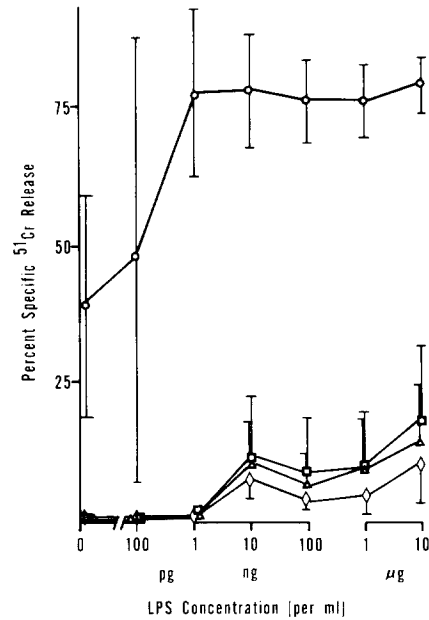
Fig. 3. Heightened LPS-responsiveness was not a systemic phenomenon in tumor-bearing mice. Increasing concentrations of LPS were added to monolayers consisting of MΦ from 7- to 14-day-old regressing Moloney sarcomas (O—O), or TG-MΦ derived from normal mice (◇—◇), or mice with 7-14 day regressing (□—□) or progressing (△—△) Moloney sarcomas. Values are the means ± standard deviations from nine separate experiments.
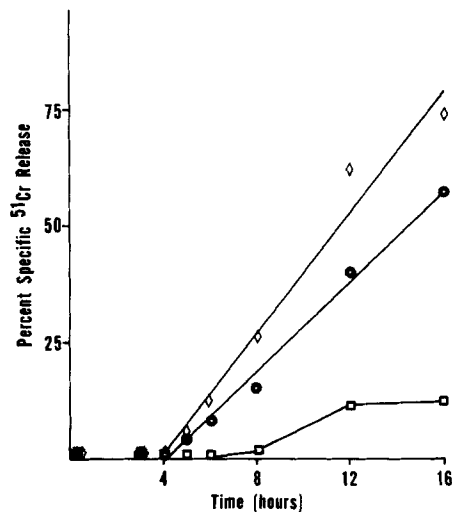

Fig. 4. The specific ^{51}Cr release mediated by MΦ obtained from 11-day-old regressing Moloney sarcomas was assayed sequentially, either with (◇—◇) or without (O—O) 100 ng LPS added/ml. First release of ^{51}Cr was detectable, though barely, 4 h after cells were introduced. Thereafter ^{51}Cr in the supernatant media increased linearly with respect to time. The rate of ^{51}Cr release was faster in LPS-stimulated cultures, accounting for the greater total amount found at 16 h, compared to unstimulated cultures. TG-MΦ from normal mice (□—□) produced an entirely different release profile at an LPS concentration (100 μg/ml) 1,000 times greater than that used to stimulate tumor MΦ; lower LPS concentrations would have had little detectable effect on TG-MΦ.

leakage of radioisotope was first (although barely) detected 4 h after mastocytoma cells were exposed to MΦ monolayers and continued to increase in a linear fashion with respect to time. The rate of ^{51}Cr release was increased with LPS present, accounting for the greater total amount of radiolabel which was found in the supernates of these cultures at 16 h.

The release profile associated with normal TG-MΦ exposed for a similar time to 1,000 times the concentration of LPS (100 μg/ml) is included in Fig. 4 to illustrate further the dramatic difference which existed between these MΦ and those derived from tumors. A longer period of time (6–8 h) was needed before TG-MΦ gave detectable release of ^{51}Cr from targets. Thereafter, radioisotope found in the supernates of cultures failed to increase in a fashion similar to that observed in wells which contained tumor MΦ.

Discussion

MΦ isolated from neoplasms may lack cytolytic activity (5–7), suggesting either that they are not fully activated or, if they are, that their lytic mechanism has been blocked in some way. The results of the studies reported here favor the first explanation. Noncytolytic tumor MΦ were shown to be only partially activated or, as we prefer to describe them, primed. No evidence was found to support the alternative possibility of a blocked effector mechanism.

The hallmark of the primed MΦ was exquisite sensitivity to stimulation by bacterial LPS: picogram to nanogram/milliliter concentrations of LPS rapidly induced primed MΦ to kill, but had no demonstrable effect on noncytolytic TG-MΦ derived either from normal or tumored mice. The finding that heightened LPS-sensitivity was not a property of TG-MΦ elicited from tumored animals indicates that priming occurs, at least in part, within the milieu of the neoplasm, rather than systemically. This finding complements and extends Hibbs' observation that full activation is a distinctly localized phenomenon (2). The primed state can thus be summarized as one in which a final signal, here supplied by LPS and perhaps given in conjunction with other stimuli (e.g., lymphokines), will trigger the expression of cytolytic activity.

The fully activated state was evanescent in culture; naturally occurring cytolytic activity of MΦ recovered from regressing Moloney sarcomas was lost within the first 24 h spent in vitro. The capacity to kill tumor cells could be regained, however, if these formerly cytolytic MΦ were exposed to minute amounts of LPS. The fact that these cells could still be induced to kill maximally by LPS after as long as 96 h in culture established that: (a) they had not reverted to the level of TG-MΦ during this period, i.e. they had remained primed; and (b) the primed state, by comparison to the fully activated one, was relatively stable.

Based on these findings we propose that MΦ in tumors proceed through at least two stages on the way to full activation. The first stage incorporates the steps which lead to priming, and apparently is completed in tumor-bearing mice regardless of whether the neoplasm in question is regressing or progressing. These MΦ cannot kill without further stimulation. Under ideal conditions, the first stage undoubtedly forms a continuum with the second, leading to full activation and the development of cytolytic activity. The signal(s) required to make this transition apparently had been given, and responded to, in sponta-

neously regressing Moloney sarcomas, as MΦ recovered from those tumors were cytolytic. MΦ recovered from two different progressing sarcomas, on the other hand, could not kill, but were primed. This finding indicated that progressor MΦ were unable to destroy tumor cells because they had failed to reach, or remain in, the fully activated state and not because there had been a complete absence of MΦ stimulation in the face of tumor progression. The fact that cytolytic MΦ could be allowed to lose their activity in vitro and then be stimulated to kill again by LPS reinforces the concept that the pathway between primed and fully activated MΦ is bidirectional and readily traversed in either direction. The long-term stability of primed MΦ in vitro, on the other hand, raises the question as to whether or not reversion to the level of the TG-MΦ is possible.

Further indirect evidence suggesting that activation is a two stage process may be found by comparing the kinetic profiles of ^{51}Cr release produced by cytolytic regressor MΦ cultured with or without nanogram/milliliter amounts of LPS, and TG-MΦ in which cytolytic activity was induced, de novo, by microgram/milliliter concentrations of LPS. The pattern of radioisotope release that characterized cytolysis mediated by tumor MΦ was similar in the presence or absence of LPS: ^{51}Cr was first detectable 4 h after prelabeled target cells were exposed to monolayers, and increased subsequently in supernates linearly with respect to time. Only the rates of ^{51}Cr release (greater in LPS-stimulated cultures) differed. The profile obtained with TG-MΦ was very different: release did not begin for 6–8 h and thereafter did not follow linear kinetics. One of us has shown, however, that a release profile essentially identical to those described here for tumor MΦ can be obtained if TG-MΦ are first preincubated with LPS for 12–16 h before cytolytic activity is measured.[2] In light of the current findings, we would view the need for preincubation and the time required to complete stage one of the activation process, i.e. to prime the TG-MΦ, as probably being one and the same.

Similar results to those described here, derived from an entirely different system, have recently been reported by Hibbs et al. (16). Rather than tumor-bearing mice, these authors used as their source of MΦ mice that had been chronically infected with either bacillus Calmette-Guérin or Toxoplasma gondii. Peritoneal MΦ elicited from these animals nonspecifically killed tumor cells, providing that fetal, rather than adult, bovine serum was used in the culture medium. The fact that killing did not occur in adult bovine serum, and was similarly undetectable in the presence of adult sera from a number of other species, led these investigators to postulate that serum factors were able to modulate the cytolytic activity of activated MΦ. Concentrations of crude endotoxin in the same range as the LPS concentrations used here were effective in overcoming the inhibition produced by adult bovine serum or, in separate experiments, by serum fractions (17). From these lines of evidence these authors hypothesized the existence of a nontumoricidal, activated MΦ, which would correspond to the noncytolytic MΦ we are describing as primed. They speculated further that nonimmunologic mechanisms in tumors might inhibit the triggering of cytolytic activity.

We have independently reached nearly the same conclusions working with

MΦ isolated from tumors; however, we differ slightly in our interpretation of the results. Both groups recognize the probable existence of agents which will trigger or augment MΦ-mediated cytolysis. In addition, Hibbs et al. (16) emphasize a negative control mechanism, i.e. one which actively suppresses the intrinsic cytolytic activity of an activated MΦ. It is here that we differ, as it is our concept that noncytolytic MΦ in tumors arise, not by subduing an otherwise cytolytic, activated MΦ, but rather due to a stimulatory defect. Such a defect could be either the lack of, or the interference with, stimulation. We contend that the primed MΦ is a stable intermediate along the path to the more labile state of full activation, and is a cell to which the activated MΦ returns *passively* upon the cessation or blocking of appropriate stimulation. Our observations that the cytolytic activity of regressor MΦ would decay in medium supplemented with FBS, but could be regained almost immediately after stimulation with small amounts of LPS, would seem to support this viewpoint.

Such relatively minor differences in interpretation will have to be resolved through additional experimentation. It can be said now, however, that the basis findings are likely to be sound, as they have been made independently by two groups working with two different systems. The fact that one of these systems utilized MΦ isolated directly from neoplasms has additionally increased the relevance these observations may have to cancer.

Summary

The state in which macrophages (MΦ) from regressing Moloney sarcomas could kill tumor target cells was a highly labile one which decayed rapidly in vitro. Thereafter, regressor MΦ were noncytolytic. MΦ from several different progressing sarcomas failed to kill, even when challenged with target cells immediately after explantation. Similarly, thioglycollate-induced peritoneal MΦ (TG-MΦ) did not kill. Noncytolytic MΦ derived either from progressing sarcomas or from long-term (up to 96 h) cultures of regressor MΦ were exquisitely sensitive to stimulation by bacterial lipopolysaccharide (LPS): picogram/milliliter amounts induced killing. Similar concentrations of LPS had no demonstrable effect on TG-MΦ. Thus, tumor MΦ generally appeared to have been primed in vivo, with those in regressing sarcomas having additionally acquired cytolytic activity. Inability of progressor MΦ to kill apparently stemmed from lack of, or failure to respond to, the signal needed in vivo to trigger cytolytic activity, rather than the total absence of activation.

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