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DEATH OF ADHERENT SPLEEN CELLS (MACROPHAGES) *in vitro* IN HYPERSENSITIVITY OF DELAYED TYPE TO MICROBIAL ANTIGENS

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The cytotoxic action of immune lymphocytes on adherent spleen cells obtained from unsensitized guinea pigs or guinea pigs sensitized with BCG was studied in autologous and allogeneic systems. The low cytotoxic effect found during culture of a suspension of spleen cells of sensitized guinea pigs with tuberculin was greatly increased after the addition of lymph node cells obtained from the same animal. Determination of death of adherent spleen cells, as also of adherent lymph node cells, can be used as a sensitive method for the detection of hypersensitivity of delayed type. The use of spleen cells as target cells is more convenient, for there are many more adherent cells in the spleen than in a suspension of lymph node cells.

KEY WORDS: *hypersensitivity of delayed type; cytotoxic action; immune lymphocytes; adherent cells.*

One of the tests for the detection of hypersensitivity of delayed type (HDP) *in vitro* is the cytotoxic action of immune lymphocytes or their supernatants (lymphotoxins) on target cells of different origins [4-7]. A previous investigation showed that lymph node cells (LNC) obtained from guinea pigs with HDT to streptococcal antigens or to tuberculin, in the presence of the specific antigen, caused death of many peritoneal exudate macrophages in monolayer culture [2]. A high percentage mortality of adherent cells (AC; macrophages) of lymph nodes also has been established in an autologous system during culture of LNC from animals sensitized by a streptococcal culture of BCG, in the presence of the specific antigen [3]. The sensitivity and specificity of this test are such that it can be used to determine HDT in experimental animals. However, the number of AC in a suspension of LNC is relatively small and if the time elapsing after sensitization is long, their number falls to 10-15 per field of vision. Meanwhile in the spleen, besides lymphocytes many AC are found.

The object of this investigation was to study whether AC of the spleen can be used as target cells for the determination of the cytotoxic effect, and also to study the cytotoxic activity of splenic lymphocytes in animals with HDT to microbial antigens.

EXPERIMENTAL METHODS

Noninbred guinea pigs were sensitized in the footpads with Freund's complete adjuvant (600 μg BCG per guinea pig). Tuberculin (from the Leningrad Institute of Vaccines and Sera) was used as the antigen in a dose of 25 μg/ml culture medium (medium No. 199 with the addition of 20% bovine serum, inactivated by heating at 56°C for 30 min, and also with 50 units penicillin and 50 μg streptomycin to 1 ml medium). The source of AC and lymphocytes was a suspension of spleen cells (SC) and LNC (inguinal, popliteal, femoral, and subclavian), taken from animals sensitized with BCG and unsensitized (control) animals. Cells obtained from each animal were washed separately in a large volume of medium No. 199 and, after centrifugation, the cell residue was resuspended in the culture medium. The suspension of LNC, after vital staining was adjusted to a concentration of $20 \cdot 10^6$ living lymphocytes/ml and divided

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into two parts. Culture medium or tuberculin was added to the cell residue after centrifugation.

After washing, the SC were resuspended in 80-ml culture medium. Part of the SC suspension was divided into several portions and again centrifuged. Either culture medium or tuberculin, or LNC with or without the addition of tuberculin was added to the cell residue up to the initial volume. The cell suspension was poured in volumes of 1 ml into test tubes. In some experiments, splenic AC, purified from lymphocytes, were used as target cells. To remove lymphocytes from the AC, the suspension of SC was poured in volumes of 1 ml into flat-bottomed test tubes (made from neutral glass), incubated for 2 h at 37°C, shaken vigorously, and the supernatant containing cells not adhering to the glass was removed. The SC remaining attached to the bottom of the tube were washed off with 1 ml fresh medium after which culture medium or tuberculin, or LNC with or without the addition of tuberculin was poured into the tubes. The suspensions of LNC and SC were kept from the time of their preparation until they were poured into the test tubes at 0°C. After culture at 37°C for 24 h and vigorous shaking, the supernatant containing nonadherent cells was removed from all the tubes and replaced by a fresh portion of culture medium (1.5 ml) without tuberculin. On the 5th-6th day after the beginning of incubation, after vital staining the number of living cells adherent to the glass, which were morphologically similar to macrophages and which were intensely stained with neutral red, was counted. Fibroblast-like cells were not counted. Counting took place in 3 or 4 tubes and the number of cells in 15 fields of vision was determined for each tube. The cytotoxic effect was determined by the equation: $CE = \frac{A - B}{A} \times 100$, where A is the mean number of cells in the control tubes (cells cultured in medium without antigen), B the mean number of cells in the experimental tubes (the same cells cultured for 24 h with antigen, and then in culture medium).

EXPERIMENTAL RESULTS

Death of AC in an autologous system during culture of SC, LNC, or a mixture of both, obtained from animals with BCG, was studied in the experiments of series I. After culture for 24 h, the formation of conglomerates consisting of macrophages surrounded by a ring of lymphocytes was observed in the experimental tubes (in the presence of tuberculin). This was particularly marked when a suspension of LNC was used, and less marked when SC was used without the addition of LNC. After incubation for 72-96 h the number of AC in the monolayer decreased appreciably. In the tubes in which adherent SC, previously purified to remove nonadherent cells, were cultured in the presence of tuberculin, the ring of lymphocytes around the macrophages was absent. The percentage mortality of the adherent LNC obtained from animals with HDT during culture with tuberculin was high. When determined on the 5th-6th day the cytotoxic effect reached 70-100% (Fig. 1A). On determination of mortality of the adherent SC after culture of the suspension of SC with specific antigen, a low cytotoxic effect (25-50%) was established (Fig. 1B). Death of AC was virtually absent if tuberculin was added to the monolayer of AC from which nonadherent cells had previously been removed (Fig. 1D). When tuberculin and LNC obtained from the same guinea pig were added to SC, the cytotoxic effect was increased by 30-60%. An increase in the percentage of death of AC was observed after the addition of LNC and tuberculin to SC, whether the latter were previously purified from lymphocytes or not (Fig. 1C, E).

The next experiments were carried out in an allogeneic system. The percentage death of adherent SC was increased by only 7-10% when LNC ($20 \cdot 10^6$) obtained from unsensitized (control) guinea pigs (a mixture from 4 animals) were added to a suspension of SC or to adherent SC from animals sensitized with BCG. A very low cytotoxic effect (10-12%) was obtained by culturing the same suspension of LNC with tuberculin. The cytotoxic effect also was determined on adherent SC of unsensitized animals with the addition of LNC obtained from animals sensitized with BCG (Fig. 2). Culture of SC of unsensitized animals, whether purified or not from nonadherent cells, led to death of virtually no AC (Fig. 2B, D). However, the addition of a suspension of LNC to animals sensitized with BCG, together with tuberculin, caused death of many of the adherent SC. The cytotoxic effect in this case exceeded 60% (Fig. 2C, E). The magnitude of the cytotoxic effect was independent of the period of sensitization (times of observation 16-80 days). Judging from the preliminary data, the cytotoxic effect evidently depends on the ratio between the number of lymphocytes and AC.

In an autologous system death of splenic AC (macrophages) of animals sensitized with BCG was found when they were cultured in the presence of the specific antigen (tuberculin). Under

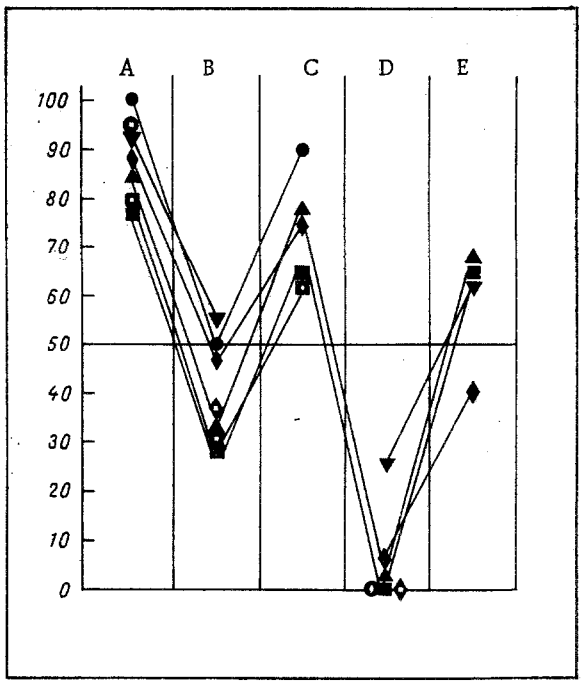

Fig. 1. Death of adherent spleen cells from sensitized animals on addition of LNC (in autologous system). A) LNC; B) suspension of SC; C) suspension of SC + LNC; D) adherent SC; E) adherent SC + LNC. Here and in Fig. 2, level of cytotoxic effect in one experiment when LNC, SC, or a mixture of both was used, is marked by identical geometric shapes. Ordinate, here and in Fig. 2, cytotoxic effect (in %).

these circumstances the cytotoxic effect was low (25-50%), suggesting an insufficient number of lymphocytes (T cells) participating in death of the autologous AC, in the spleen of animals sensitized by the method described above. One piece of evidence in support of this conclusion is the increase in the cytotoxic effect up to 60-80% on the addition of LNC obtained from animals with HDT to the original SC suspension. It was also shown that the number of T cells in the lymph nodes was almost twice that in the spleen [8]. The possibility likewise cannot be ruled out that the potential activity of the T cells of the spleen and lymph nodes, when this method of sensitization was used, was not identical.

Death of AC (macrophages) from the lymph nodes and spleen in an autologous system in the presence of the specific antigen may perhaps take place in HDT not only *in vitro*, but also *in vivo*. The ensuing liberation of cytoplasmic and lysosomal enzymes from the macrophages may evidently play a role in the injury to the surrounding tissues in immunopathological processes connected with HDT [1].

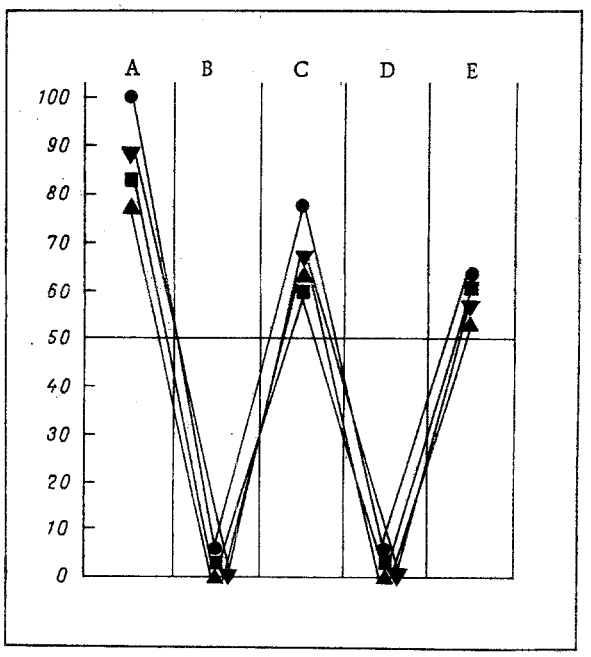

Fig. 2. Death of adherent spleen cells of unsensitized animals on additon of LNC from animals sensitized with BCG (in allogeneic system). A) LNC(s); B) suspension of SC(c); C) suspension of SC(c) + LNC(s); D) adherent SC(c); E) adherent SC(c) + LNC(s). s) Cells of sensitized animals; c) cells of unsensitized (control animals.

Determination of the percentage mortality among the AC used in this sytem as the target cells may serve as a sensitive method for the detection of HDT in experimental animals. The use of SC as the source of AC is more convenient, for AC are much more numerous in the spleen than in a suspension of LNC.

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