# IMMUNOLOGICAL TOLERANCE IN VITRO: KINETIC STUDIES AT THE CELLULAR LEVEL*

BY E. DIENER, PH.D., AND W. D. ARMSTRONG,‡ M.D.

*(From The Walter & Eliza Hall Institute of Medical Research,§ Melbourne 3050, Australia)*

(Received for publication 21 October 1968)

There is indirect experimental evidence suggesting that the state of immunological tolerance is a central failure of the immune system to initiate immunological reactions against a particular antigen and that the main lesion in tolerance is to be sought at the level of the antigen-reactive lymphocyte (1). From work on the specificity of antigenic determinants in hapten-carrier systems (2–4), on feedback inhibition mechanisms depending on competition for antigenic determinants by 19S and 7S antibodies (5), and on lymphocyte stimulation by anti-allotype serum (6), it would seem that the participation of immune competent cells in an immune reaction depends on the interaction of surface-bound recognition sites and antigenic determinants. Since immunological tolerance is antigen specific, it is self-evident that similar basic mechanisms must determine the induction of unresponsiveness at the cellular level.

New experimental possibilities regarding the cellular and molecular mechanisms which lead to the state of immunological tolerance have emerged with the introduction of a system which not only allows the tolerization of mouse spleen cells to a protein antigen in vitro, but also the subsequent testing on the cellular level (14) for the degree of unresponsiveness by in vitro antigenic challenge of the cultured cells (7). Such a system bears the advantage over in vivo models in that it depends on the interaction between a defined number of cells and an antigen which can be quantitated accurately and which is distributed homogenously throughout the cell suspension in culture.

Using this in vitro technique combined with an in vivo method for the enumeration of antigen reactive cells (8), the present study provides evidence that immunological tolerance induced in vitro to the protein polymer of *Salmonella adelaide* flagellin reduces the number of immune competent cells reactive to this antigen. Furthermore, experiments on the kinetics of tolerance

* This work was supported by the National Health & Medical Research Council, Canberra (Australia), the U.S. Atomic Energy Commission (AT-20-1-3695), and the National Institute of Allergy and Infectious Diseases, U.S. (AI-0-3958).

‡ Supported by a Medical Research Council of Canada Fellowship. Present address: Department of Pathology, Faculty of Medicine, University of Alberta, Edmonton, Alberta, Canada.

§ This is publication number 1292 from The Walter & Eliza Hall Institute.

induction with respect to different parameters, such as antigen dose and duration of exposure of cells to a tolerogenic dose of antigen at different temperatures, suggest that this phenomenon must be primarily related to the interaction between the surface of immunologically competent cells and antigen molecules.

## *Materials and Methods*

*Cell Cultures.*—The tissue culture method used was patterned on that described by Marbrook (9) and modified according to Diener and Armstrong (7). A 2 ml suspension of spleen cells in tissue culture medium containing an appropriate amount of antigen was placed in a cylindrical tube, the bottom end of which was closed off by a dialysis membrane. This tube was then placed into an Erlenmeyer-type flask containing an adequate volume of tissue culture medium (approximately 50–70 ml), to equal the fluid level of the cell suspension. Cultures were placed in a humidified incubator at 37°C with a constant flow of gas consisting of 10% $CO_2$, 7% $O_2$, and 83% $N_2$ (10).

*Tissue Culture Medium.*—Balanced salt solution was prepared by adding to 100 ml of double distilled water 5.2 g NaCl, 400 mg KCl, 200 mg $CaCl_2$, 200 mg $MgCl_2 \cdot 6H_2O$, 150 mg Na $HPO_4 \cdot 2H_2O$, and 1 g glucose.

Concentrated minimal essential medium (Eagle's) was prepared by mixing 10 ml amino acids (solution A, 100 × concentrated) with 10 ml amino acids (solution B, 100 × concentrated), 10 ml 200 mM glutamine (100 × concentrated), and 10 ml vitamins (100 × concentrated) (Commonwealth Serum Laboratory, Australia). The solution was supplemented with 110 mg of sodium pyruvate and 21 mg of serine. The balanced salt solution was then mixed with the concentrated medium and 1 ml of 1% phenol red, 10,000 IU penicillin, and 50,000 IU streptomycin were added. The solution was sterilized by Millipore filtration (0.45 $\mu$) and thereafter supplemented with 160 ml of sterile 2.8% w/v $NaHCO_3$ and 100 ml of sterile fetal calf serum (Commonwealth Serum Laboratory, Australia). Double distilled water was added to give a final volume of 1 liter of medium.

*Cell Suspensions Prepared from Spleens.*—Normal and immunized $CBA_{T6T6}$ male or female mice, 70–110 days old were used as the source of spleen cells. After cervical dislocation, their spleens were removed, finely minced, and strained through a stainless steel wire mesh into the tissue culture medium using sterile technique. Cells were counted in a hemocytometer and cell viability was tested by the eosin dye-exclusion test (11). On the average, $25 \times 10^6$ spleen cells were placed in 2 ml of medium for each tissue culture.

*Antigen.*—Polymer was prepared from flagellin of *S. adelaide* and *S. waycross* (H antigens) as described by Ada, Nossal, Pye, and Abbott (12). Sterile antigen was prepared by filtering the flagellin preparation through a Millipore membrane (0.45 $\mu$ pore size) before polymerization. The sterile antigen was diluted in doubly distilled water containing 0.1% fetal calf serum and frozen in aliquots for storage. Sheep erythrocytes were washed in normal saline and resuspended in medium at a concentration of $2 \times 10^7$ cells per ml. Iodination of antigen was by the chloramine T method, using carrier free preparations of $^{125}I$ (Radiochemical Centre, Amersham, England), as described elsewhere (13). Substitution rates varied from 0.01–0.06 g atom of iodide per 30,000 g of flagella (0.7–4 $\mu c/\mu g$).

*Bacteria.*—*Salmonella derby:* (H antigen fg; O antigen 1, 4, 12) was used as the indicator strain for enumeration of single antibody-forming cells (14) to *S. adelaide* (H antigen fg; O antigen 35). *S. derby* shares the H but not the O antigen with *S. adelaide*. For enumeration of antibody-forming cells to *S. waycross*, (H antigen: Z4, Z23; O antigen 41), the indicator strain used was *S. cerro*, (H antigen: Z4, Z23; O antigen 18). *S. cerro* shares the H but not the O antigens with *S. waycross*. The organisms were grown in nutrient broth (12).

*Assay for Enumeration of Antibody-forming Cells.*—The cell suspension was collected sepa-

rately from each culture, washed twice, and assayed for the number of antibody-forming cells, using the adherence colony method described by Diener (14) for the enumeration of cells secreting antibody against polymer, and the hemolysin microplaque technique of Cunningham (15) to assess the number of cells secreting antibody against sheep erythrocytes.

*Treatment of Cells with Tolerogenic and Immunogenic Doses of Antigen at a Temperature of 4°C.*—Spleen cell suspensions at a concentration of $12 \times 10^6$ cells per 1 ml of fluid were kept in 25 ml Erlenmeyer flasks on ice and the pH of the suspending medium was adjusted in a gas flow consisting of 10% $CO_2$, 7% $O_2$, and 83% $N_2$ to pH 7.3. After an appropriate amount of antigen was added to the suspensions, the containers were sealed with parafilm, and transferred into a refrigerator where they were kept for various lengths of time at a temperature of 4°C. After such treatment, the suspensions were centrifuged at 500 *g* for 7 min, the cells resuspended in an appropriate volume of fresh medium, and centrifuged again. After adequate washing, the cells were placed in tissue culture flasks at a concentration of $12 \times 10^6$ cells per 1 ml of medium.

*Assay for the Enumeration of Antigen Reactive Cells.*—Antigen-reactive cells may be defined as cells which respond to an antigen by differentiation and proliferation into a progeny of antibody forming cells. The number of such cells present in cell suspensions after in vitro culture was determined using the technique described in detail by Armstrong and Diener (8, 16). This method makes use of Salmonella H antigens and is patterned after a system designed for the enumeration of cells reacting to sheep erythrocytes (17, 18). Cells cultured for 4 days in vitro were harvested and a fixed number injected intravenously into lethally irradiated syngeneic hosts. The following day 25 $\mu$g of polymer from *S. adelaide* flagellin was injected into the irradiated recipients to stimulate antigen reactive donor cells which had seeded into the host's spleen to elicit the production of antibody-forming cell populations. 7 days later, the animals were sacrificed, their spleens removed, and cut into slices 260 $\mu$ thick which were placed in order on a microscope slide coated with a thin layer of *S. derby* bacteria suspended in "motility agar" (0.6% gelatine and 0.4% shredded agar in heart infusion broth) at 42°C. The spleen slices were then covered with a layer of sterile "motility agar" and incubated at 37°C for 2½ hr to allow the bacteria from the lower layer of the slide to move to the upper layer of clear agar. The preparations were then kept for a further 15–18 hr at 20°C during which time the bacteria grew into a thick film. Areas of antibody production in the spleen slices were detected by the absence of bacterial growth characterized by a clearly visible translucent focus in the agar. It has been shown previously (8, 16) that there is a linear relationship between the number of cells injected into the recipient and the number of foci (antigen reactive cell-focus, ARC) produced in its spleen, and this is taken as an indication that each focus is due to a cluster of cells derived from a single antigen-reactive unit.

*Irradiation.*—Mice were placed in groups of 10 in plastic containers and irradiated with a dose of 850 rad. A Philips (R.T. 250) 250 kev machine was used and irradiation was performed at 15 ma and a half-value layer (HVL) of 0.8 mm Cu. To maintain adequate temperature, humidity, and breathing conditions for the mice during irradiation, the plastic container was flushed with a constant flow of air at a rate of ½ liter per min.

## RESULTS

*Antigen Dose Relationship of the In Vitro Immune Response to Polymer of S. adelaide Flagellin.*—When spleen cultures from normal $CBA_{T6T6}$ mice were stimulated with different doses of polymer from *S. adelaide* flagellin (POL) ranging from 0.2 ng[1] to 3 $\mu$g[2] per ml of culture fluid it was found that the peak

[1] Microgram ($\mu$g, $10^{-6}$ g).

[2] Nanogram (ng, $10^{-9}$ g).

number of antibody-forming cells (AFC) by day 4 was antigen dose–dependent, 2–20 ng/ml of POL being the optimal concentration. However, as the amount of antigen was increased, a sharp fall in the number of AFC was noted, and when 2–3 $\mu$g/ml of POL was added to the cultures, virtually no immune response was detected (Fig. 1). In order to test for the specificity of this unresponsiveness, $4 \times 10^6$ sheep red blood cells (SRBC) were added to the tissue

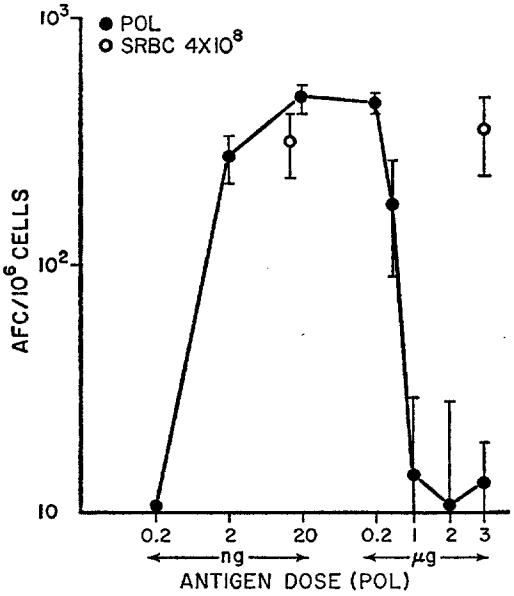


FIG. 1. Effect of increasing doses of antigen, polymer (*POL*) of *S. adelaide* flagellin on in vitro immune response. Cell suspensions tested for the number of antibody-forming cells at 4 days of culture. Vertical bars indicate 95% confidence limits. ●——●, arithmetic mean number of antibody-forming cells (*AFC*) to *POL* per $10^6$ harvested cells. O, arithmetic mean number of *AFC* to $4 \times 10^6$ sheep red blood cells (*SRBC*) per $10^6$ harvested cells. Each value comprises the result obtained from 15 to 100 cultures.

culture flasks together with 3 $\mu$g/ml of POL. When these cultures were assayed 4 days later for the number of AFC to the two antigens it was found that the spleen cells had retained their ability to become immune to SRBC (350 AFC/$10^6$ harvested cells), but were unable to produce an immune response against POL (Fig. 1). Similarily, the addition of an immunogenic dose of POL from *S. waycross* along with a tolerogenic concentration of POL from *S. adelaide* resulted in an average response of $249 \pm 83$ AFC/$10^6$ harvested cells to *S. waycross* in the absence of AFC to *S. adelaide*. Cultures stimulated with *S. waycross* only gave an average response of $265 \pm 72$ AFC/$10^6$ harvested cells. To test for concomitant immunity during the early stages of tolerance induc-

tion, cell suspensions cultured with a tolerogenic dose of POL were tested for the presence of AFC at 2 and 3 days after beginning the cultures. At none of these chosen time points was there any sign of an immune reaction.

Based on the phenomenon of two zones of antigen dosage which could produce immunological tolerance in vivo (19, 20), an attempt was made to demonstrate the same effect at the single cell level in vitro. Cultures were set up for 12 hr without antigen and with different doses of POL, ranging from 2 pg[3] to 0.2 ng/ml. Subsequent to this treatment, an optimally immunogenic concentration of 20 ng/ml of POL was added to each culture. Assessment of AFC was carried out after 4 days of culture. Such a time course if applied to the treatment of cells with 3 μg/ml of POL would have resulted in tolerance

TABLE I

*Immune Response of Spleen Cells In Vitro to Challenge with an Immunogenic Dose of POL after Preincubation for 12 Hr with Subimmunogenic Concentrations of Antigen**

| Antigen concentration during preincubation of cells for 12 hr | Antigen concentration for challenge | Immune response in terms of mean numbers of AFC/$10^6$ harvested cells by day 4 |
|---|---|---|
| | *ng* | |
| 20 ng | 20 | 777 ± 186 |
| 0.2 ng | 20 | 832 ± 344 |
| 0.02 ng | 20 | 502 ± 173 |
| 2 pg | 20 | 226 ± 176 |
| No antigen | 20 | 205 ± 28 |

* Each group comprises results obtained from 10 cultures.

(7). Instead, preincubation of cells for 12 hr with antigen doses too low to be immunogenic (Fig. 1) resulted in a normal 4 day immune response when followed by an optimally immunogenic dose for 3½ days. This effect became less pronounced as the preincubation dose was reduced and finally reached the level of the control group which was preincubated for 12 hr without antigen and subsequently stimulated with an optimally immunogenic concentration of POL for 3½ days (Table I).

*The Number of Immunologically Competent Cells in Spleen Cell Suspensions Cultured with Immunogenic and Tolerogenic Amounts of Antigen.*—In order to test for the presence or absence of cells immunologically reactive to POL (antigen-reactive cells, ARC) the method by Armstrong and Diener (8) for the enumeration of ARC was applied to spleen cell cultures. Cells kept in vitro for 4 days either without antigen or in the presence of 20 ng/ml or 3 μg/ml of POL respectively were harvested and injected intravenously into lethally irradiated syngeneic recipients at doses of 2 to 5.6 × $10^6$ viable cells per mouse. The

[3] Picogram (pg, $10^{-12}$ g).

mice were stimulated with 25 μg of POL the following day, an antigen dose which is optimally immunogenic in vivo. 7 days later, the spleen of each recipient was removed and assayed for the number of antigen-reactive cell foci (ARC foci). The results recorded in Table II indicate that the adoptive immune response of tolerant cells as measured by the "per cent specific activity" (per cent value of immunologically active spleen slices within a whole sequence of slices tested) is 10% of the response of those cells given an immunogenic dose of POL in vitro and 20% of the response produced by cells which were cultured in the absence of antigen. Based on the nonparametric rank test, the differences are significant at a $P$ value of $<0.01$.

*Kinetics of Tolerance Induction as Related to the Time of Incubation of Cells with Tolerogenic Doses of Antigen at a Temperature of 37°C.*—As seen from Fig. 1,

TABLE II

*Adoptive In Vivo Immune Response of Mouse Spleen Cells* Made Immune or Tolerant In Vitro to Polymer (POL) of S. adelaide Flagellin*

Response measured at the level of the antigen-reactive cell (ARC).

| In vitro treatment for 4 days | Viable cultured cells injected | Mean specific activity |
|---|---|---|
| No antigen | $2.5 \times 10^6$ or $5 \times 10^6$ | 10.5 |
| 20 ng POL/ml | $2 \times 10^6$ | 34 |
| 3 μg POL/ml | $2.5 \times 10^6$ or $5.6 \times 10^6$ | 2.3 |

* Each group comprises results obtained from 16 to 19 recipients.

the minimal concentration of antigen required to induce immunological tolerance in vitro is in the order of 2–3 μg per ml of culture fluid. For assessment of the kinetics of tolerance induction as related to the duration of treatment of spleen cells with antigen, it was decided to use 30 μg/ml of POL as the tolerogenic stimulus, since the application of 3 μg/ml to cells for less than 12 hr gave somewhat irregular results. Suspensions of $25 \times 10^6$ spleen cells/ml were incubated either in the presence of 30 μg/ml or 20 ng/ml of antigen for time intervals of 15 min, 1, 3, and 6 hr at a temperature of 37°C. After such time, the cells were removed from the culture vessels, washed twice in culture medium equivalent in volume to that used for incubation, and finally cultured in the presence of 20 ng/ml of POL for 4 days. In order to test for toxicity of the antigen during the 6 hr preincubation of cells in the presence of 30 μg/ml of POL, $2 \times 10^6$ SRBC/ml were added to the cultures, and the resulting immune response compared with adequate control groups. No significant difference was found regarding the hemolysin response elicited by the two groups, the values being $115 \pm 80$ AFC/$10^6$ harvested cells to SRBC in the presence of 20 ng of POL and $111 \pm 53$ AFC/$10^6$ harvested cells to SRBC when 30 μg of POL were added for 6 hr to the cultures along with SRBC. Results regarding the degree of

tolerance induced to POL relative to the time of preincubation of spleen cells with tolerogenic amounts of the antigen are shown in (Fig. 2). Partial tolerance had been induced to a significant degree after preincubation of spleen cells with 30 μg/ml of POL for a period of 15 min only ($P = <0.01$). This is indicated by a 50% yield in the number of AFC after pretreatment with the tolerogenic antigen dose of cells, as compared with the relevant control value. As the time

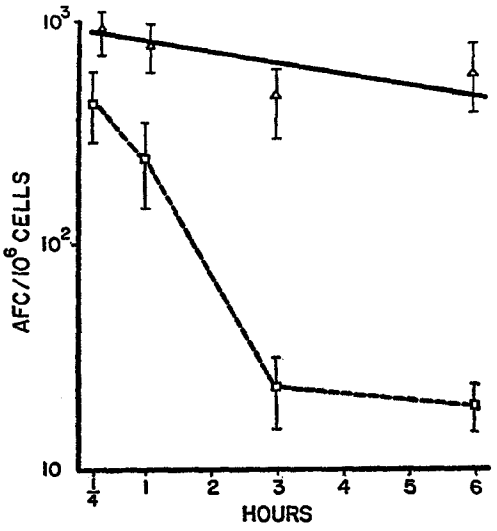


FIG. 2. Induction of immunological tolerance to polymer of *S. adelaide* flagellin: preincubation of cells at 37°C with 20 ng and 30 μg of antigen for time intervals of ¼, 1, 3, and 6 hr followed by culture with 20 ng for 4 days. Vertical bars indicate 95% confidence limits. △——△, arithmetic mean number of antibody-forming cells (*AFC*) per $10^6$ harvested cells. Preincubation with 20 ng/ml of antigen. □----□, arithmetic mean number of *AFC* per $10^6$ harvested cells. Preincubation with 30 μg/ml of antigen. Each value comprises the results obtained from 10 to 15 cultures.

interval of preincubation for tolerance induction was increased, the immune responses became progressively weaker, indicating that virtually complete tolerance was established by exposing the cells to 30 μg/ml of POL for a minimal time of 3 hr.

*Kinetics of Tolerance Induction as Related to the Time of Incubation of Cells with Tolerogenic Doses of Antigen at a Temperature of 4°C.*—Particularly interesting results were obtained when spleen cells were mixed with 30 μg/ml and 20 ng/ml of POL, held at 4°C for time intervals ranging from 1 to 6 hr and then washed twice and cultured with the immunogenic dose of 20 ng/ml of POL at 37°C for 4 days. It is shown in Fig. 3 that no significant degree of tolerance was obtained when the exposure time of cells to 30 μg/ml of POL

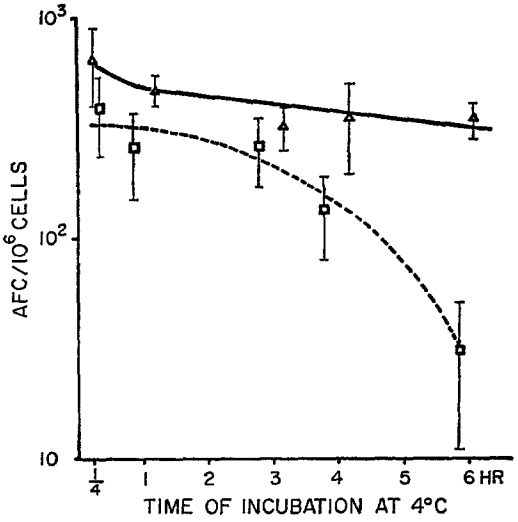


FIG. 3. Induction of immunological tolerance to polymer of *S. adelaide* flagellin: preincubation of cells at 4°C with 20 ng and 30 μg of antigen for time intervals of ¼, 1, 3, 4, and 6 hrs, followed by culture with 20 ng at 37°C for 4 days. △——△, arithmetic mean number of antibody-forming cells (*AFC*) per $10^6$ harvested cells. Preincubation with 20 ng/ml of antigen. □----□, arithmetic mean number of antibody-forming cells (*AFC*) per $10^6$ harvested cells. Preincubation with 30 μg/ml of antigen. Each value comprises the results obtained from 10–30 cultures.

TABLE III

*Influence of the Washing Procedure on the Carry-Over of Antigen after Preincubation of Cells with Radio-Labeled Polymer of S. adelaide Flagellin*

After each wash, cells were resuspended in tissue culture medium to a concentration of approximately 12 × $10^6$ cells/ml.

| Antigen concentration per ml | Time of exposure to POL | Temperature during exposure | Antigen carry-over after the: | | | |
|---|---|---|---|---|---|---|
| | | | 1st wash | 2nd wash | 3rd wash | 4th wash |
| | | °C | % | % | % | % |
| 20 ng | 6 hr | 4 | 0.8 | 0.30 | 0.10 | 0.10 |
| 3.00 μg | 6 hr | 4 | 0.4 | 0.10 | 0.08 | 0.03 |
| 30.00 μg | 6 hr | 4 | 0.5 | 0.09 | 0.09 | 0.05 |
| 20 ng | 6 hr | 37 | 0.9 | 0.2 | | |
| 3.00 μg | | | | | | |
| 30 μg | 15 min | 4 | 0.3 | 0.07 | 0.06 | 0.06 |

was kept within 15 min–4 hr, but when tolerance induction in the cold was allowed to proceed for 6 hr, a tolerance effect was provoked. This is demonstrated by over a 10-fold difference in the yield of AFC in the tolerant group as compared with the relevant controls.

*Tests to Quantitate Carry-Over of Antigen after Preincubation of Cell Cultures with Different Concentrations of POL.*—Studies were performed using $^{125}$I-labeled POL to determine the amount of antigen carried over by cells after preincubation with various antigen concentrations. Results shown in Table III indicate that separation of the cells by centrifugation from the antigen containing medium already reduced their contamination with POL to levels of less than 1% of the original concentration. Furthermore, no significant difference could be found in the residual amount of antigen carried over by cells, with respect to either the different antigen concentrations used, or the temperature and duration of preincubation. On the basis of this investigation, it was calculated that, under the conditions used in the experiments, viz., preincubation of spleen cells with 30 μg/ml POL, followed by two washes, the antigen carry-over was well within the immunogenic range.

## DISCUSSION

In these experiments, use has been made of a technique which allows the induction of immunity and tolerance of mouse spleen cells in vitro under conditions which enable the accurate control of the dose of antigen and the timing of its application. It was thus possible to increase or reduce the antigen concentration of the cell cultures at will and it was shown that residual amounts of antigen after antigen removal by adequate washing of the cells had no detectable influence on the experimental results obtained.

Based on the justified assumption that the focus assay for the enumeration of antigen-reactive cells (8, 16) provides a direct quantitative reflection of the immune potential to *Salmonella* H antigens of lymphoid cells, our data indicate that the number or reactivity of antigen-reactive cells is greatly diminished in spleen cells cultured with 30 μg/ml of antigen in vitro. Furthermore, the quantitative assessment of such cells in an adoptive in vivo response system has served as a highly sensitive test to prove the validity of what was believed to be tolerance induced in vitro. In addition to results published previously (7) on the specificity of in vitro tolerance, it has been shown that unresponsiveness can be induced in cell cultures to *S. adelaide* H antigens at concentrations of 3 and 30 μg/ml along with unimpaired immunity to both sheep erythrocytes and H antigens from *S. waycross* which are not cross reactive with *S. adelaide*.

It is known from tolerance experiments in vivo, that a stage of immunity precedes the state of tolerance (21–23). This phenomenon is believed to be due to "terminal differentiation" of initially immunized cells, which temporarily

mask the tolerization of newly recruited immunologically competent cells (24). The present investigation lends no support to the concept outlined above. It is likely that concomitant immunity during the initial phase of tolerance induction in vivo is the reflection of a heterogeneous distribution of antigen within the lymphoid system leading to both immunity and tolerance according to differences of the local antigen concentration. In contrast, the in vitro system guarantees an even distribution of a soluble antigen throughout a fixed number of cells and thus allows the exposure of the relevant target cells to equal quantities of antigen and hence leads to a more uniform cellular reaction.

One of the striking features of successful tolerance induction is its dependence on the antigen dose used for induction. This is most clearly demonstrated by there only being a 10-fold difference in the dose of polymer present in the cultures which determines either tolerance or immunity. This, taken together with the finding that tolerance can be induced to a significant degree within 15 min exposure of spleen cells to tolerogenic doses of POL in vitro, strongly suggests the state of unresponsiveness at the single cell level to be the result of a direct and specific encounter of a threshold number of antigen molecules with the relevant target cell. Such a concept does not negate the increasing evidence that the immune response to some antigens may specifically be mediated at least by more than one cell class (25). It may be that the primary target cell upon which tolerance is enforced is not even the precursor of antibody-forming cells, but that blockage of such a cell by a tolerogenic dose of antigen would interrupt the succession of cell to cell interaction processes necessary for immunity. Experiments on transformation of small lymphocytes into blast cells (6) and on allotypic suppression (26, 27) have provided increasing evidence that the discrimination of antigenic determinants is mediated by recognition sites at the surface of immunologically competent cells. It is thought that such a mechanism would result in the attachment of antigen molecules to the cell's recognition sites. Based on the previously made assumption that there may be a threshold quantity of antigen responsible for successful tolerance induction, it follows that this threshold must be determined by the probability of antigen molecules encountering recognition sites. Such a mechanism would depend solely on laws of mass action and thus should occur at temperatures at which the cell metabolism is minimal. Our findings on induction of tolerance by exposure of spleen cells in the cold to tolerogenic concentrations of antigen provide strong support for such a concept. Our data however do not give evidence for the mechanism of action of the cell attached antigen, which finally causes the tolerization of the cell concerned. The demonstration of two zones of antigen concentration which could produce immunological tolerance in vivo (19, 20) has prompted us to look for the same effect in vitro. Using similar conditions as for high zone tolerance induction, except for the antigen concentra-

tion which in this case was kept at subimmunogenic levels, we were not able to demonstrate low zone tolerance in vitro. This indicates the possibility that tolerance in vivo to repeatedly administered subimmunogenic doses of antigen is exerted by specialized structures lacking in a single cell suspension and these structures accumulate antigen to concentrations comparable to those necessary for tolerance induction in vitro. Such a theory based on a series of convincing experimental facts has been proposed (28).

It was the purpose of this paper to concentrate on the basic mechanisms at the cellular level which underlie the phenomenon of immunological tolerance, and it is hoped that this approach may provide a means of interpreting some of the more complex tolerance results gained from in vivo experiments.

## SUMMARY

When normal mouse spleen cells in suspension are cultured in vitro in the presence of polymer from *S. adelaide* flagellin, an immune response can be obtained as measured at the level of single antibody-forming cells. Cultures were stimulated with different doses of antigen, ranging from 0.2 ng to 3 $\mu$g/ml of tissue culture fluid and it was found that the peak number of approximately 500 antibody-forming cells per $10^6$ harvested cells by day 4 was antigen dose dependent, 2–20 ng/ml being the optimal concentration. When more than 1 $\mu$g/ml of polymer from *S. adelaide* together with either 20 ng/ml of polymer from *S. waycross* or with $4 \times 10^6$ sheep erythrocytes were placed in the system, unresponsiveness to *S. adelaide*, but immunity to the other antigens occurred simultaneously. Cells made immunologically tolerant in vitro to *S. adelaide* H antigens were transferred into syngeneic lethally irradiated recipients and challenged with the same antigen. The adoptive immune capacity in these mice, as measured at the level of the immunologically competent cell was reduced by 80–90% as compared with relevant controls. Attempts to induce low zone tolerance in vitro were without success. To study the kinetics of tolerance induction in vitro, cells were cultured with tolerogenic doses of antigen for various periods of time, washed, and subsequently cultured with immunogenic doses of antigen for 4 days. It was found, that immunological tolerance may be induced to a significant degree in vitro within a period of 15 min. Similar results were obtained when spleen cells were exposed for various lengths of time to tolerogenic doses of antigen but at a temperature of 4°C instead of 37°C. The results are taken as suggestive evidence that the initial step in tolerance induction is related to the direct interaction between the surface of immune competent cells and antigen molecules.

We wish to thank Professor G. J. V. Nossal for his support and interest, and Dr. G. Ada for supplying the antigen. The excellent technical assistance of Mrs. J. Coghlin and of Misses J. Burkitt, P. Harris, and M. Bakker is gratefully acknowledged.

## BIBLIOGRAPHY


1. McGregor, D. D., P. J. McCullagh, and J. L. Gowans. 1967. The role of lymphocytes in antibody formation: Restoration of the haemolysin response in X-irradiated rats with lymphocytes from normal and immunologically tolerant donors. *Proc. Roy. Soc. Ser. B. Biol. Sci.* **168**:229.
2. Green, I., W. E. Paul, and B. Benacerraf. 1967. Hapten carrier relationship in the DNP-PLL foreign albumin complex system: Induction of tolerance and stimulation of cells *in vitro*. *J. Exp. Med.* **126**:959.
3. Mitchison, N. A. 1967. Antigen recognition responsible for the induction *in vitro* of the secondary response. *Cold Spring Harbor Symp. Quant. Biol.* **32**:431.
4. Rajewsky, K., E. Rottlaender, G. Peltre, and B. Mueller. 1967. The immune response to a hybrid protein molecule: Specificity of secondary stimulation and of tolerance induction. *J. Exp. Med.* **126**:581.
5. Henry, C., and N. K. Jerne. 1968. Competition of 19S and 7S antigen receptors in the regulation of the primary immune response. *J. Exp. Med.* **128**:133.
6. Sell, S., and P. G. H. Gell. 1965. Studies on rabbit lymphocytes *in vitro*: I. Stimulation of blast formation with an anti-allotype serum. *J. Exp. Med.* **122**:423.
7. Diener, E., and W. D. Armstrong. 1967. Induction of antibody formation and tolerance *in vitro* to a purified protein antigen. *Lancet.* **2**:1281.
8. Armstrong, W. D., and E. Diener. A new method for the enumeration of antigen-reactive cells responsive to a purified protein antigen. *J. Exp. Med.* **129**:371.
9. Marbrook, J. 1967. Primary immune response in cultures of spleen cells. *Lancet.* **2**:1279.
10. Michell, R. I., and R. W. Dutton. 1966. Immunization of normal mouse spleen cell suspensions *in vitro*. *Science.* **153**:1004.
11. Hanks, J. H., and J. H. Wallace. 1950. Determination of cell viability. *Proc. Soc. Exp. Biol. Med.* **98**:188.
12. Ada, G. L., G. J. V. Nossal, J. Pye and A. Abbot. 1964. Antigens in immunity: I. Preparation and properties of flagellar antigens from *Salmonella adelaide*. *Aust. J. Exp. Biol. Med. Sci.* **42**:267.
13. Ada, G. L., G. J. V. Nossal, and J. Pye. 1964. Antigens in immunity. III. Distribution of iodinated antigens following injection into rats via the hind footpads. *Aust. J. Exp. Biol. Med. Sci.* **42**:295.
14. Diener, E. 1968. A new method for the enumeration of single antibody-producing cells. *J. Immunol.* **100**:1062.
15. Cunningham, A. J. 1965. A method of increased sensitivity for detecting single antibody-forming cells. *Nature.* **207**:1106.
16. Diener, E., W. D. Armstrong, W. A. Robinson, and J. Marbrook. 1968. New methods to study the kinetics of the immune response. *Biochem. Pharmacol. Suppl.* 63.
17. Kennedy, J. C., L. Sminovitch, J. E. Till, and E. A. McCulloch. A transplantation assay for mouse cells responsive to antigenic stimulation by sheep erythrocytes. *Proc. Soc. Exp. Biol. Med.* **120**:868.
18. Playfair, J. H. L., B. W. Papermaster, and L. J. Cole. 1965. Focal antibody production by transferred spleen cells in irradiated mice. *Science.* **149**:998.

19. Mitchison, N. A. 1964. Induction of immunological paralysis in two zones of dosage. *Proc. Roy. Soc. Ser. B. Biol. Sci.* **161**:275.
20. Shellam, G. R. and G. J. V. Nossal. 1968. Mechanism of induction of immunological tolerance: IV. The effects of ultra-low doses of flagellin. *Immunology.* **14**: 273.
21. Brent, L., and G. Gowland. 1962. Induction of tolerance of skin homografts in immunologically competent mice. *Nature.* **196**:1298.
22. Siskind, G. W., and J. G. Howard. 1966. Studies on the induction of immunological unresponsiveness to pneumococcal polysaccharide in mice. *J. Exp. Med.* **124**: 417.
23. Nossal, G. J. V., and G. M. Austin. 1966. Mechanism of induction of immunological tolerance: II. Simultaneous development of priming and tolerance. *Aust. J. Exp. Biol. Med. Sci.* **44**:327.
24. Sterzl, J. 1966. Immunological tolerance as the result of terminal differentiation of immunologically competent cells. *Nature.* **209**:416.
25. Mitchell, G. F., and J. F. A. P. Miller. 1968. Immunological activity of thymus and thoracic duct lymphocytes. *Proc. Nat. Acad. Sci. U.S.* **59**:296.
26. Dubiski, S., and K. Fradette. 1966. The feed-back mechanism in immunoglobulin synthesis. *Proc. Soc. Exp. Biol. Med.* **122**:126.
27. Mage, R., and S. Dray. 1965. Persistent altered phenotypic expression of allelic γG-immunoglobulin allotypes in heterozygous rabbits exposed to isoantibodies in fetal and neonatal life. *J. Immunol.* **95**:525.
28. Ada, G. L., and C. R. Parish. 1968. Low zone tolerance to bacterial flagellin in adult rats. A possible role in antigen localized in lymphoid follicles. *Proc. Nat. Acad. Sci.* In press.