Vaccination against Experimental Autoimmune Diseases Using T Lymphocytes Treated with Hydrostatic Pressure

OFER LIDER,[a] MEIR SHINITZKY,[b] AND IRUN R. COHEN[a]

[a]*Department of Cell Biology*
[b]*Department of Membrane Research*
The Weizmann Institute of Science
Rehovot, 76100 Israel

BACKGROUND

Diseases thought to involve autoimmune processes include multiple sclerosis, rheumatoid and other arthritides, type I diabetes mellitus, and various forms of thyroiditis. In these diseases, the particular target organ suffers irreparable damage from progressive or repeated insults by the individual's immune system. At present, there exists no specific therapy for the pathogenic process of these conditions. Nonspecific anti-inflammatory drugs are used symptomatically in arthritis, exogenous hormones are administered to replace endogenous insulin or thyroid hormones, and indiscriminant immunosuppression is used as a last resort in severe cases of multiple sclerosis or rheumatoid arthritis. (It is also used experimentally in early type I diabetes.) An ideal mode of therapy would be one that could selectively nullify those autoreactive lymphocytes responsible for the disease, while leaving intact the other, healthy components of the immune system.

The laboratory of one of us (I.R.C.) has initiated steps in the direction of this goal by deploying lines and clones of autoimmune T lymphocytes to vaccinate animals against particular experimental autoimmune diseases.[1] The strategy was to isolate and grow in long-term culture the T lymphocytes that caused autoimmune diseases in rats or mice. These include experimental autoimmune encephalomyelitis (EAE),[2,3] experimental autoimmune thyroiditis (EAT),[4] and adjuvant arthritis (AA).[5-7] The T-lymphocyte lines have been used successfully to investigate factors important in disease: identification of the target antigens of autoimmune attack,[8,9] migration of the T lymphocytes to their target organ,[10] their persistence in the body,[11] their pathogenic effects *in vivo*[12] and *in vitro*,[13] and their expression of enzymes[14] and surface markers[10] associated with function.

Relevant to the present communication was the observation that autoimmune T lymphocytes could induce resistance, that is, vaccinate animals against the specific disease. Rat T-lymphocyte lines reactive to the basic protein of myelin (anti-BP) will, upon intravenous inoculation, produce EAE in naive rats.[2,3] However, the virulence of the anti-BP line cells could be attenuated by irradiating them (1500R). Such line cells could no longer produce EAE, but rats receiving these attenuated cells acquired resistance to EAE induced later by active immunization to BP in complete Freund's adjuvant (BP/CFA).[10,15,16] Vaccination with irradiated T lymphocytes, though, has

deficiencies; there was little resistance to EAE produced by passive transfer of virulent anti-BP line cells[17] and individual clones of the anti-BP line were incapable of vaccination.[18]

Anti-thyroglobulin (anti-Tg) T-lymphocyte lines were found to mediate EAT in mice.[4] Attenuated anti-Tg T lymphocytes vaccinated mice against EAT, produced either actively by immunization to Tg/CFA or passively by inoculation with virulent anti-Tg line cells.[4]

Adjuvant arthritis, unlike EAE or EAT, is not induced by immunization to a defined self-antigen, but by immunization to antigens of *Mycobacterium tuberculosis* (MT).[19] By raising rat T lymphocytes reactive to MT, we isolated a clone, A2b, that produced arthritis in heavily irradiated (750R) rats.[5,6] This arthritogenic clone responded *in vitro* both to MT and to a fraction of the proteoglycan of joint cartilage.[8] Thus, AA probably is caused by T lymphocytes that recognize an epitope of MT cross-reactive with rat joint cartilage. Clone A2b was not able to vaccinate rats against AA.[6] However, another clone, A2c, was not arthritogenic and the rats receiving A2c acquired resistance to AA (in preparation). Although the process of vaccination remains to be clarified, it is reasonable to suspect that the autoimmune receptors of the T-lymphocyte vaccine induce resistance to disease by activating antireceptor immunity.[1,20] If this is so, then procedures that increase the immunogenicity of the T-lymphocyte receptor might increase the potency of vaccination induced by T-lymphocyte line cells.

AUGMENTATION OF IMMUNOGENICITY BY HYDROSTATIC PRESSURE

The rationale behind our strategy for increasing the potency of vaccination by autoimmune T lymphocytes was derived from observations related to augmentation of immunogenicity of tumor cells. The laboratory of one of us (M.S.) has been investigating nonadversative means for augmentation of immunogenicity of tumor cells through lateral and vertical rearrangement of their membrane proteins (reviewed in reference 21). Currently two types of vaccines have been prepared from tumor cells: cholesterol-treated cells and pressure-treated cells. Pressure treatment of tumor cells was found to be very effective in enhancing the immunogenicity of tumor-associated antigens.[22] We therefore undertook to investigate whether hydrostatic pressure similarly would augment the potency of autoimmune T lymphocytes as vaccines against autoimmune disease.

PRESSURE TREATMENT INHIBITS PROLIFERATION OF T LYMPHOCYTES

T-lymphocyte lines treated with the pressure used to enhance the immunogenicity of tumor cells (1.2–1.5 kbar for 15 min) were found to remain intact and they excluded the vital dye, trypan blue (not shown). However, as shown in TABLE 1, pressure-treated line cells were unable to proliferate *in vitro* when stimulated by incubation with specific antigen or the T-cell mitogen, Concanavalin A. Irradiation with 1500R left the lymphocytes with a negligibly small response, yet with an apparent normal morphology. However, death and disintegration of both irradiated and pressure-treated line cells were observed after culture for 3–4 days (not shown). Thus, pressure rendered the T lymphocytes unresponsive and moribund.

TABLE 1. T-Lymphocyte Lines Are Unable to Proliferate after Treatment with Pressure[a]

T Lymphocyte Line		Proliferative Response (cpm × 10^{-3} ± SD)			
Specificity	Treatment	No. Antigen	BP	MT	Con A
Anti-BP	none	0.9 ± 0.2	115 ± 3	0.7 ± 0.3	140 ± 7
	irradiation	0.9 ± 0.1	2.3 ± 0.4	1.0 ± 0.2	2.5 ± 0.5
	pressure	0.4 ± 0.2	0.6 ± 0.2	0.4 ± 0.1	0.7 ± 0.3
Anti-MT	none	1.5 ± 0.4	0.9 ± 0.3	98.3 ± 1	138 ± 7
	irradiation	1.1 ± 0.5	1.2 ± 0.2	3.0 ± 0.3	2.9 ± 0.2
	pressure	0.8 ± 0.2	0.6 ± 0.2	0.7 ± 0.1	0.6 ± 0.3

[a]Anti-BP[3] and anti-MT lines[5] were developed and maintained as described. The line cells were irradiated as described[3] or treated with pressure as follows. The cells were suspended in PBS at a concentration of 7×10^7/ml and placed in a sterile Eppendorf centrifuge tube. A 22G needle was inserted through the cap of the tube and the tube was filled with cold PBS (0°C) and sealed. The tube was then placed in a cooled (4°C) pressure cylinder (American Instrument, Aminco., Silver Spring, Maryland) and the cylinder was introduced into a French Press. Pressure was applied over 7–8 min to a level of 1.5 kbars, where it was maintained for 15 min and then released over another 7–8 min. The pressure-treated cells were washed in PBS and used in a proliferative response assay as described.[3] Results are shown as the cpm of incorporated ^{3}H-thymidine. Each well of a microtiter plate contained 25×10^3 line cells, 2×10^6 irradiated Lewis thymocytes (1500R), and 10 μg/ml of BP or MT, or 2.5 μg/ml Con A. Each group was cultured in quadruplicate for 72 hours. ^{3}H-Thymidine was added for the last 12 hours of incubation. (BP = myelin basic protein, MT = *Mycobacterium tuberculosis*, irradiation = 1500R, pressure = 1.5 kbar.)

TABLE 2. Pressure Treatment of Lines or Clones Augments Vaccination against EAE[a]

				Vaccination	
				% Inhibition of EAE Induced by:	
Anti-BP Lymphocytes	BP Activation	Treatment	% Incidence of Passive EAE	BP/CFA	Anti-BP Line
Line Z1a	no	none	0	0	0
	yes	none	100	70	10
	yes	irradiation	0	70	0
	no	pressure	0	0	0
	yes	pressure	0	80	100
Clone D9	no	none	0	0	0
	yes	none	100	0	0
	yes	irradiation	0	0	0
	no	pressure	0	0	0
	yes	pressure	0	80	100

[a]Line Z1a and clone D9, both reactive against the 68–88 peptide of BP,[18] were activated or not by incubation with BP and irradiated accessory cells as described.[10] Line and clone cells were treated with irradiation[3] or pressure as described in the footnote to TABLE 1. Lewis rats (5–15 per group) were inoculated intravenously with 10^7 T lymphocytes as described[3] and the incidence of EAE mediated by the T lymphocytes was recorded. Rats to be tested for vaccination were inoculated with 2×10^7 treated T lymphocytes intraperitoneally in incomplete Freund's adjuvant. Thirty days later, the rats were challenged with BP/CFA to induce active EAE or with 5×10^6 Z1a line cells to induce passive EAE. (Irradiation = 1500R; pressure = 1.5 kbar, 15 min.)[3]

PRESSURE-TREATED T LYMPHOCYTES VACCINATE

TABLE 2 summarizes the effects of anti-BP line Z1a and clone D9 in producing EAE or vaccinating against EAE. Line Z1a, if not activated by incubation with BP before inoculation, neither caused EAE nor vaccinated against it.[20] Activated anti-BP line cells produced EAE in all recipient rats and those rats that recovered from acute disease showed resistance to active EAE induced by immunization to BA/CFA. However, there was little resistance to EAE mediated by a second injection of the Z1a line itself. As was reported, activated and irradiated line cells did not produce EAE, but recipient rats were vaccinated against active EAE.[10,15] However, these rats were not resistant to passive EAE produced by the anti-BP line.[17] In contrast, inoculation of rats with pressure-treated, activated anti-BP line cells induced resistance to both active and passive EAE. Moreover, the pressure-treated cells themselves did not produce EAE. Pressure-treated, nonactivated anti-BP line lymphocytes, though, did not vaccinate.

The results with anti-BP clone D9 were even more striking. Similar to the other anti-BP clones, D9 could only produce EAE, but could not vaccinate against either active or passive disease.[18] Nevertheless, activated anti-BP clone D9, when treated with pressure, vaccinated recipient rats against both active EAE and EAE produced by the Z1a line. Thus, pressure rendered anti-BP T lymphocytes nonvirulent and as superior vaccines against EAE.

VACCINATION IS SPECIFIC

The results of experiments designed to test the specificity of vaccination with anti-BP line cells are shown in TABLE 3. It can be seen that anti-BP line cells activated by Con A were as effective as those activated by BP in vaccinating rats against EAE. Thus, acquired resistance could not be explained by carryover of BP into the recipient rats. This conclusion was further supported by the failure of anti-MT line cells or normal thymocytes to vaccinate against EAE even after they had been cultured together with BP.

In additional experiments, we found that pressure-treated anti-BP line cells did not vaccinate against AA and that pressure-activated anti-MT line cells vaccinated rats

TABLE 3. Specificity of Vaccination[a]

Pressure-Treated Cells	Activation	Vaccination: % Inhibition of EAE Induced by: BP/CFA	Vaccination: % Inhibition of EAE Induced by: Anti-BP Line
Anti-BP	BP	73	93
Anti-BP	Con A	75	100
Anti-BP	none	0	0
Anti-MT	MT	0	0
Anti-MT	MT + BP	0	0
Thymocytes	BP	0	0

[a]Anti-BP[3] or anti-MT[5] line cells or Lewis thymocytes were activated by incubation with antigens or Con A as described. The cells were then treated with pressure as described in the footnote to TABLE 1. Rats were inoculated with 2×10^7 cells in incomplete Freund's adjuvant intraperitoneally, and one month later were challenged to induce EAE actively with BP/CFA or passively with 5×10^6 anti-BP line cells.[3]

TABLE 4. Pressure-Treated Anti-BP Lymphocytes Must Be Intact to Vaccinate[a]

Activated Anti-BP Line	Treatment	Subsequent EAE via Anti-BP Line		
		Incidence	Clinical Score	% Protection
none	none	15/15	4	0
yes	irradiation	5/5	2.8	0
yes	P	1/15	0.2	93
yes	P + sonication	3/3	3	0
yes	P membrane	3/3	3	0
yes	cholesterol	4/4	3.5	0

[a]Anti-BP line cells were activated as described[3] and rats were incubated intraperitoneally with 2×10^7 cells that had been irradiated (1500R) or treated with pressure (see footnote to TABLE 1). Some rats were inoculated with an equivalent number of pressure-treated cells (P) that had been disrupted by sonication or with membrane preparations of pressure-treated cells. Sonication was done using an Ultrasonic Model W325 at intervals of 5 sec × 8. Membranes were prepared by homogenizing the cells and centrifuging the homogenate in a 41% sucrose gradient at 90,000G. Cholesterol packing of cells was done using a solution of cholesterol hemisuccinate in a PVP solution. The clinical score was determined as follows: 1 = tail weakness; 2 = paralysis of hind limbs; 3 = paralysis of all limbs; 4 = moribund. (Pressure = 1.5 kbar, 15 min; irradiation = 1500R.)

against AA, but not against EAE (not shown). Thus, vaccination was immunologically specific and could not be attributed to transfer of antigen.

VACCINATION REQUIRES INTACT T LYMPHOCYTES

Experiments were done to learn whether augmented vaccination required that the pressure-treated anti-BP line cells be intact. TABLE 4 shows that pressure-treated, but not irradiated, activated anti-BP line cells vaccinated rats against passive EAE. However, sonication of the pressure-treated cells or membrane preparations of these cells failed to vaccinate. Enriching the anti-BP cells with cholesterol also failed to render them effective vaccines. Thus, the ability of pressure-treated T lymphocytes to induce resistance to disease appears to depend on the intactness of the cells. Moreover, augmented vaccination cannot be attributed to membrane ridigification alone as treatment with cholesterol had no effect.

MECHANISM OF VACCINATION

Two important questions must be answered: What are the critical effects of pressure on the T lymphocytes that render them vaccines and what are the mechanisms of resistance that they induce in the recipient? Hydrostatic pressure operates exclusively on compressible compartments, and in intact cells, these are comprised of the membrane lipid layer and the cytoskeletal polymers.[21] While under pressure in the range used in our study, the lipid layer becomes significantly more rigid and the cytoskeletal network practically disintegrates. These changes lead to aggregations of membrane proteins that are largely preserved after the pressure is released, and the lipid layer and the cytoskeletal return to their normal state.[21] Examination of the cell surface using a fluorescent antibody assay indeed indicated that the pressure treatment produced permanent aggregation of lymphocyte surface major histocompatibility class

I antigens (not shown) and Thy1, 2.[22] We suspect that similar aggregation of the T lymphocyte antigen receptor may also take place as a result of the pressure treatment. These antigen aggregates presumably increase considerably the immunogenic expression of the T-cell receptors. As the pressure-treated T lymphocytes are nonresponsive and moribund, it is not likely that they participate actively in the process of resistance. Vaccination, therefore, is probably accomplished by the response of the recipient to the rearranged membrane antigens of the inoculated T lymphocytes.

If, indeed, resistance is due to antireceptor immunity, then that immunity is unexpectedly comprehensive since a single clone can induce resistance to disease produced either by an uncloned line or by active immunization. Either all anti-BP T lymphocytes share a common receptor idiotype or immunization to a single receptor idiotype generates a mechanism that recognizes the antigen and suppresses all the receptor-bearing effector T lymphocytes that also see the antigen, irrespective of their idiotype. These possibilities are being explored. Whatever the mechanism turns out to be, it is now evident that pressure-treated T lymphocytes are a powerful tool for exciting resistance to experimentally induced autoimmune disease. The ultimate question is the feasibility of this strategy to produce resistance in clinical autoimmunity.

REFERENCES

1. COHEN, I. R., A. BEN-NUN, J. HOLOSHITZ, R. MARON & R. ZERUBAVEL. 1983. Vaccination against autoimmune disease using lines of autoimmune T lymphocyte. Immunol. Today **4:** 227–230.
2. BEN-NUN, A., H. WEKERLE & I. R. COHEN. 1981. The rapid isolation of clonable antigen-specific T lymphocyte lines capable of mediating autoimmune encephalomyelitis. Eur. J. Immunol. **11:** 195–199.
3. BEN-NUN, A. & I. R. COHEN. 1982. Experimental autoimmune encephalomyelitis (EAE) medicated by T cell lines: Process of selection of lines and characterization of the cells. J. Immunol. **129:** 303–308.
4. MARON, R., R. ZERUBAVEL, A. FRIEDMAN & I. R. COHEN. 1983. T lymphocyte line specific for thyroglobulin produces or vaccinates against autoimmune thyroiditis in mice. J. Immunol. **131:** 2316–2322.
5. HOLOSHITZ, J., Y. NAPARSTEK, A. BEN-NUN & I. R. COHEN. 1983. Lines of T lymphocytes induce or vaccinate against autoimmune arthritis. Science **219:** 56–58.
6. HOLOSHITZ, J., A. MATITIAU & I. R. COHEN. 1984. Arthritis induced in rats by cloned T lymphocytes responsive to mycobacteria, but not to collagen type II. J. Clin. Invest. **73:** 211–215.
7. HOLOSHITZ, J., A. MATITIAU & I. R. COHEN. 1985. Role of the thymus in induction and transfer of vaccination against adjuvant arthritis with a T lymphocyte line in rats. J. Clin. Invest. **75:** 472–477.
8. VAN EDEN, W., J. HOLOSHITZ, Z. NEVO, A. FRENKEL, A. KLAJMAN & I. R. COHEN. 1985. Arthritis induced by a T lymphocyte clone that responds to *Mycobacterium tuberculosis* and to cartilage proteoglycans. Proc. Natl. Acad. Sci. USA. **82:** 5117–5120.
9. COHEN, I. R., J. HOLOSHITZ, W. VAN EDEN & A. FRENKEL. 1985. T lymphocyte clones illuminate pathogenesis and effect therapy of experimental arthritis. Arthritis Rheum. **28:** 841–845.
10. NAPARSTEK, Y., A. BEN-NUN, J. HOLOSHITZ, T. RESHEF, A. FRENKEL, M. ROSENBERG & I. R. COHEN. 1983. T lymphocyte lines producing or vaccinating against autoimmune encephalomyelitis (EAE): Functional activation induces PNA receptors and accumulation in the brain and thymus of line cells. Eur. J. Immunol. **13:** 418–423.
11. NAPARSTEK, Y., Y. HOLOSHITZ, S. EISENSTEIN, T. RESHEF, S. RAPPAPORT, S. CHEMKE, A. BEN-NUN & I. R. COHEN. 1982. Effector T lymphocyte line cells migrate to the thymus and persist there. Nature **300:** 262–264.

12. HOLOSHITZ, J., Y. NAPARSTEK, A. BEN-NUN, P. MARQUARDT & I. R. COHEN. 1984. T lymphocyte lines induce autoimmune encephalomyelitis delayed hypersensitive and bystander encephalitis or arthritis. Eur. J. Immunol. **14:** 729–732.
13. YAROM, Y., Y. NAPARSTEK, V. LEV-RAM, J. HOLOSHITZ, A. BEN-NUN & I. R. COHEN. 1983. Immunospecific inhibition of nerve conduction in isolated rat optic nerve exerted by a line of T lymphocytes reactive to basic protein of myelin. Nature **303:** 246–247.
14. NAPARSTEK, Y., I. R. COHEN, Z. FUKS & I. VLODAVSKY. 1984. Activated T lymphocytes produce a matrix degrading heparan sulphate endoglycosidase. Nature **310:** 241–244.
15. BEN-NUN, A., A. WEKERLE & I. R. COHEN. 1981. Vaccination against autoimmune encephalomyelitis with T lymphocyte line cells reactive against myelin basic protein. Nature (London) **292:** 60–61.
16. HOLOSHITZ, J., A. FRENKEL, A. BEN-NUN & I. R. COHEN. 1983. Autoimmune encephalomyelitis (EAE) mediated or prevented by T lymphocyte lines directed against diverse antigenic determinants of myelin basic protein. Vaccination is determinant specific. J. Immunol. **131:** 2810–2813.
17. BEN-NUN, A. & I. R. COHEN. 1981. Vaccination against autoimmune encephalomyelitis (EAE). Attenuated autoimmune T lymphocytes confer resistance to induction of active EAE, but not to EAE mediated by intact T lymphocyte line. Eur. J. Immunol. **11:** 949–952.
18. VANDENBARK, A. A., H. OFFNER, T. RESHEF, R. FRITZ, C-H. J. CHOU & I. R. COHEN. 1985. Specificity of T lymphocyte lines for peptides of myelin basic protein. J. Immunol. **135:** 229–233.
19. PEARSON, C. M. 1964. Experimental models in rheumatoid disease. Arthritis Rheum. **7:** 80–86.
20. COHEN, I. R. 1984. Autoimmunity: Physiologic and pernicious. Adv. Intern. Med. **29:** 147–165.
21. SHINITZKY, M. 1984. Membrane fluidity in malignancy—adversative and recuperative. Biochim. Biophys. Acta **738:** 251–261.
22. RICHERT, L., A. OR & M. SHINITZKY. Promotion of tumor immunogenicity in EL4 cells subjected to hydrostatic pressure. Cancer Immunol. Immunotherapy. In press.

DISCUSSION OF THE PAPER

I. R. MACKAY (*Royal Melbourne Hospital, Victoria, Australia*): Have you followed cutaneous DTH reactions in parallel with the induction and suppression of disease? It appears from the time intervals that the apparent helper cells that you are injecting are in fact acting as effectors.

I. R. COHEN: (*Weizmann Institute of Science, Rehovot, Israel*): We have looked at DTH and these cells do, if they are activated, transfer DTH to their specific antigen.