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Development of autoimmune process in rats immunized with influenza vaccine

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Background. The role of vaccination in the development of autoimmunity has been extensively discussed in the literature; nevertheless, it remains vague. Whether there is a causal relationship between vaccination and the mechanisms leading to the autoimmune phenomena has yet to be discovered. Therefore, characterization of autoimmune disease in Wistar rats immunized with influenza vaccine was the basis of the current report.

Materials and methods. Forty-three Wistar male rats (30 with AA and 13 healthy) were used. Two groups of animals with adjuvant arthritis (AA) and one group of healthy rats were immunized intramuscularly (i.m.) four times with 0.01 ml of influenza vaccine. Prophylactic and therapeutic vaccinations were made. Untreated groups (with AA and healthy) served as controls. Body and organ weight, blood and pro/antioxidant system indices in serum, joint swelling, development of polyarthritis, as well as histological changes in joints, liver and lungs were evaluated.

Results. A more aggressive pathological process with elevated ESR and MDA levels and histological changes in the lungs and liver were observed in vaccinated animals. Despite a significant joint swelling and changes in periarticular soft tissues (increase of inflammatory infiltration, edema and angiomatosis), changes in the synovium and cartilage of vaccinated animals did not differ from those in AA controls, although the therapeutic vaccination induced more pronounced edema and inflammatory infiltration with granulocytes and macrophages in synovium, fissures (in 30% of animals), enhanced the rise of erosium (by 13.9%), usures (by 31.6%) and the thinning of cartilage (by 25%).

We failed to detect any evidence of a clinically observed autoimmune process in healthy rats after vaccination, except only minimal histological changes in joints of some animals.

Conclusions. Our preliminary findings are the first to show the presence of a certain relationship between vaccination and the exacerbation of autoimmune disease. The use of commercial influenza vaccine enhances the autoimmune process expressed by increased joint swelling and changes in soft periarticular tissues of experimental animals, and induces changes in the lungs. A greater impairment was observed after therapeutic vaccination.

Key words: rats, adjuvant arthritis, vaccination

INTRODUCTION

Vaccines are the most cost-effective intervention in terms of reducing the burden of disease and ultimately death in both influenza pandemics and epidemics (1). Influenza is a major killer and world health problem. Infection rates are highest in children, but complication rates are highest in the elderly, patients with chronic pulmonary and cardiovascular diseases, immunosuppressed patients, such as those suffering from rheumatoid arthritis (RA), and diabetics. Widespread vaccination, particularly of individuals at a high risk, is crucial in decreasing incidence, controlling epidemics, and reducing complications (2).

Although the role of vaccination in the development of autoimmunity has been extensively discussed in the literature, it remains vague. Whether there is a causal relationship between vaccination and the mechanisms leading to the autoimmune phenomena has yet to be discovered (3). The idea that infectious agents and viruses could trigger the development of autoimmune diseases in genetically susceptible individuals has been raised in various studies (3, 4) and attracted considerable interest. However, clear criteria are required to establish a causative role for infectious agents and vaccines in a disease process.

Vaccines contain peptides that are homologous to viral antigens, therefore, it is not surprising that the question of

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whether vaccination could lead to autoimmune illness has risen in many scientific debates (3, 5). Vaccines are known to induce an immune response similarly to infections and may trigger, just like infections do, autoimmune diseases, chronic fatigue syndrome (CFS) and fibromialgia (6). Furthermore, vaccines contain an adjuvant which enhances their immune stimulation. Aluminum adjuvants have been widely used in vaccines for more than 60 years (7). Although other adjuvants have also proved to be effective (8), the aluminum adjuvant is the only nonproprietary alternative for influenza vaccine manufacturers (9–11). However, there is no conclusive evidence for a causal link between vaccination and the development of autoimmune diseases (12). Analysis of disease mechanisms can be greatly aided if animal models are available or might be developed.

In the literature, there are medical and some animal studies relating to a wide range of autoimmune diseases temporally associated with vaccination (13). However, the lack of studies with animals has hampered basic research aimed to understand an interrelation between vaccination and the development of autoimmune features. Animal models that have been used to study the immunogenicity of vaccines include mice, rats, guinea pigs and rabbits (14). Mice – regardless of breed, age, or method of injection – are poor responders to vaccines, and the response in guinea pigs is highly variable among animals. Some authors note that mice and rats provide a useful small-animal model for the study of influenza virus pathogenesis (15–19). Although the mouse is a commonly used preclinical model for studying the immunogenicity of influenza vaccines by using the same vaccine dose and administration routes as in humans (1), the response in rats is less variable among individual animals, making this animal the most suitable model in immunogenicity studies (20).

It should be noted that no animal models have been adequately standardized and validated to predict the risk of autoimmunity associated with vaccines (21). However, a number of existing models can be considered for use, but they need refinement to be applied to vaccine evaluation.

A desirable reference standard would be an animal model of vaccine-enhanced autoimmune disease. In order to allow the elucidation of such problem, an animal model of autoimmune disease – adjuvant arthritis (AA) – was used. Characterization of autoimmune disease in Wistar rats immunized with influenza vaccine was the basis of the current report.

MATERIALS AND METHODS

Animals

A total of 43 young adult male Wistar rats (10–12 weeks old), weighing 180–230 g were obtained from the Institute of Immunology (Vilnius, Lithuania) and maintained in the vivarium of the Institute of Experimental and Clinical Medicine at Vilnius University. The animals were housed in large plastic cages (7–10 per cage) with the diet of standard rodent chow and water, at 20–22 °C and 50–70% relative humidity with 10–12 h light / dark cycle. Theyhad been acclimated for 7 days before experiments. Throughout the study, the animals were cared for in accordance with the European Convention and Guide for the Care and Use of Laboratory Animals and with Lithuanian laws. All experiments were performed using protocols approved by the Lithuanian Laboratory Animal Use Ethics Committee under the State Food and Veterinary Service.

Vaccines and reagents

The commercial human Influenza vaccine (Influvac 2007 / 2008 formulation, Solvay Pharmaceuticals; *Vaccinum influenzae inactivatum ex cortices antigeniis praeparatum* [(15 μg + 15 μg + 15 μg) / 0.5 ml]) was used. Complete Freund's adjuvant (CFA), 10% formalin, spirit formol, hematoxylin-eosin, picrofuxin, toluidine blue, methyl-green-pyronin-9, safranin O, orthophosphoric acid, thiobarbituric acid, nitric acid, ferrous sulfate, ascorbic acid, ammonium molybdate, hydrogen peroxide were obtained from Sigma-Aldrich Chemie and Fluka Chemie GmbH (Germany).

Induction of adjuvant arthritis and clinical evaluation of disease

AA was induced in 30 rats by the foot pad method, i. e. injection of 0.1 ml of CFA into the left hind paw under light anaesthesia with Apaurin (KRKA). Each AA group contained 10 animals. The course of AA was assessed on the grounds of paw swelling, development of polyarthritis and histological changes in the soft periarticular tissues, synovium and cartilage. Animals were observed three times a week, and their joint swelling and body weight were monitored. The swelling of hind paws and the development of polyarthritis in three non-injected paws were determined plethysmographically by using PVP1001 (Kent Scientific Corporation). The experiment lasted 35 days.

Immunization of rats with AA

One-fifth of the commercial single human dose (SHD; 0.5 ml) of vaccine was diluted in sodium saline, and the animals were immunized intramuscularly with 0.01 ml of SHD. Intramuscular injections (i. m. i.) employed a 30-Ga needle and 1-ml syringe (BD) and were given into the quadriceps muscle. Group I rats with AA served as an untreated control and were injected with saline. Group II rats with induced AA received influenza vaccine on days 1, 8, 15, and 30 (prophylactic vaccination) and group III on days 8, 15, 21, and 30 (therapeutic vaccination). Group IV of 8 healthy animals was vaccinated on days 1, 8, 15, and 30. The interval among the first three vaccinations was 7 days. The fourth last vaccination was made two weeks later in the prophylactic treatment and 9 days later in the therapeutic treatment. The total dose was 0.04 ml of SHD. Five days after the last vaccination, i. e. on day 35 of the experiment, the rats were sacrificed.

Blood and tissue collection

The animals were monitored until day 35 when they were humanely killed by decapitation under a light narcosis. Their internal organs were examined macroscopically and weighed. The erythrocyte and leukocyte counts (made using a Picoscale, Hungary) and the erythrocyte sedimentation rate (ESR) were determined in their blood. The obtained indices were compared with the indices of normal (healthy) non-vaccinated and vaccinated animals. The liver, lungs, and joints were used for further histological analysis. Blood samples were centrifuged at 800 g for 10 min to obtain serum samples which were stored frozen at –20 °C until testing.

Determination of lipid peroxidation (MDA), catalase and total antioxidant activity (AOA) levels in blood serum

The end product of lipid peroxidation (MDA), the antioxidant enzyme catalase and the total antioxidant activity (AOA) were determined in the blood serum of all test groups. The healthy non-vaccinated and vaccinated rats served as controls.

The MDA level in blood serum, expressed as nmol per mg protein, was determined by the thiobarbituric acid reaction at 535 nm and 580 nm by the method of Gavrilov and coworkers (22) which is the modified method of Ohkawa et al. (23) and is used until now (24).

Catalase activity, expressed in $mmol \cdot min^{-1} \cdot mg$ tissue, was measured at 410 nm as described by Koroliuk et al. (25), which is the modified method of Aebi et al. (26).

The total antioxidant activity was determined in the reaction with thiobarbituric acid, described by Galaktionova et al. (27) and expressed as the percentage of reduction to control values.

Histopathology of the liver, lungs and joints

The liver, lungs and the joints of adjuvant-injected paws were collected for histological examination. Ankle joints were fixed in 10% formalin and liver samples in spirit-formol. Lungs were inflated with 10% neutral buffered formalin to their normal volume and then immersed in the same fixative solution. Following decalcification in 10% nitric acid (HNO_3) and paraffin embedding, the specimens of joints were cut on a microtome into multiple levels. Histological sections of joints and liver were stained with hematoxylin-eosin (for visualization of cells), picrofuxin (for determination of fibrotic processes and collagen fibres), toluidine blue (for visualization of proteoglycan loss and cartilage damage), methyl-green-pyronin-9 (plasmatization), and safranin 0 (for evaluation of changes in the cartilage) and reviewed using light microscopy. Lung sections were stained with haematoxylin and eosin (H & E), and evaluated for inflammation and epithelial damage. Four parameters of pulmonary inflammatory changes were scored in each lung section: peribronchiolitis (bronchocentral granuloma, inflammatory cells clustered around the periphery of small airways, peribronchial fibrosis), interstitial damage (interstitial pneumonia associated with inflammatory cells and rising of rheumatoid granuloma with inflammatory cell infiltration), alveolar (hemorrahages and inflammatory infiltration) and vascular damage (fibrinoid necrosis, sclerosis, perivascular fibrosis). Histological assessment of inflammatory infiltration with lymphocytes, plasma cells, macrophages and granulocytes and of various other inflammatory symptoms in liver, lungs, synovium and soft periarticular tissues as well as evaluation of cartilage damage were performed in a blinded manner by a pathologist on a 0–3 scale, where 0 indicates the absence of changes and 3 means the most severe expression of a particular symptom.

Statistics

All data were expressed as mean ± SEM. Statistical analysis was done using SPSS / PC software version 8.0 using t test statistics for continuous variables, and P values less than 0.05 were considered to be significant. The nonparametric Mann–Whitney test was used for evaluation of histological changes in the test organs.

RESULTS

Organs and blood indices

To estimate the impact of vaccination on systemic inflammation, for each animal the body weight and the weight of internal organs was determined, as well as blood indices were measured and compared with the indices of healthy vaccinated animals at the end of the study. Twenty rats with AA and 8 healthy rats were vaccinated four times with Influvac.

Prophylactic vaccination of arthritic rats induced a significant decrease of the total body weight since day 19 till the end of experiment as compared with healthy vaccinated animals ($P < 0.05–0.01$). The body weight of therapeutically vaccinated rats was also significantly lower than that of healthy vaccinated animals since day 19 till day 32 ($P < 0.05–0.02$). However, no significant differences between the control AA group and both arthritic vaccinated groups of animals were found. In healthy vaccinated rats, the total body weight was higher than in the control AA group since day 19 till day 32 ($P < 0.05–0.02$) (data not shown).

The average absolute and relative weight of the organs at the end of experiment is shown in Table 1. Postmortem examination of internal organs in the control and all vaccinated groups revealed a significantly higher relative weight of the liver, kidney, and spleen ($P < 0.01–0.001$) in comparison with healthy animals. The relative weight of the spleen also significantly differed from that in the healthy vaccinated group ($P < 0.001$). A decrease of absolute and relative thymus weight was observed in both vaccinated AA groups versus healthy vaccinated ($P < 0.05–0.001$) and non-vaccinated ($P < 0.01–0.001$) groups. A significant difference from the control AA group was revealed in all vaccinated groups in which the absolute and the relative weight of the spleen

Table **1. Body and absolute and relative weight of organs in healthy and arthritic rats immunized with influenza vaccine**

Index		Groups				
		I (AA control) n = 10	II (AA + prophylactic vaccination) n = 10	III (AA + therapeutic vaccination) n = 10	IV (healthy vaccinated animals) n = 8	V (healthy animals) n = 5
Body weight (g)		280.50 ± 5.73*	273.77 ± 6.54*+	279.70 ± 5.73*	305.07 ± 10.67	309.00 ± 10.17
Liver (g)	Absolute	10.78 ± 0.32	10.08 ± 0.48+	10.84 ± 0.25	11.62 ± 0.49	10.34 ± 0.40
	Relative	3.84 ± 0.05*	3.94 ± 0.13*	3.88 ± 0.06*	3.80 ± 0.05*	3.34 ± 0.06
Kidneys (g)	Absolute	2.30 ± 0.08*	2.24 ± 0.06	2.32 ± 0.05*	2.39 ± 0.11*	2.06 ± 0.06
	Relative	0.82 ± 0.02*	0.82 ± 0.02*	0.83 ± 0.01*	0.78 ± 0.03*	0.66 ± 0.01
Spleen (g)	Absolute	1.05 ± 0.05*	•1.25 ± 0.058*+	•1.53 ± 0.08*+	•0.80 ± 0.04*	0.62 ± 0.02
	Relative	0.37 ± 0.015*	•0.46 ± 0.02*+	•0.48 ± 0.03*+	•0.26 ± 0.008*	0.20 ± 0.004
Lung (g)	Absolute	2.29 ± 0.18	2.19 ± 0.11	2.11 ± 0.18	2.16 ± 0.12	–
	Relative	0.82 ± 0.07	0.80 ± 0.04	0.76 ± 0.07	0.71 ± 0.03	–
Thymus (g)	Absolute	0.50 ± 0.04*	0.46 ± 0.037*+	0.57 ± 0.04*+	•0.70 ± 0.046	0.84 ± 0. 05
	Relative	0.18 ± 0.015*	0.169 ± 0.014*+	0.20 ± 0.01*+	•0.23 ± 0.01	0.27 ± 0.02

Note. Adjuvant arthritis (AA) was induced in 30 rats by the foot pad method by injecting 0.1 ml of CFA into the left hind paw under light anaesthesia. Group I (control) – animals with induced AA. Second two groups – rats with AA immunized intramuscularly (i. m.) four times on days 1, 8, 15, and 30 in prophylactic vaccination (group II) and on days 8, 15, 21, and 30 in therapeutic vaccination (group III) with 0.01 ml of single human dose (SHD) of vaccine (total 0.04 ml of SHD). Group IV – 8 healthy animals immunized with influenza vaccine on days 1, 8, 15, and 30. The differences are significant in comparison with healthy not vaccinated (*) and vaccinated (+) animals and with the control group (•).

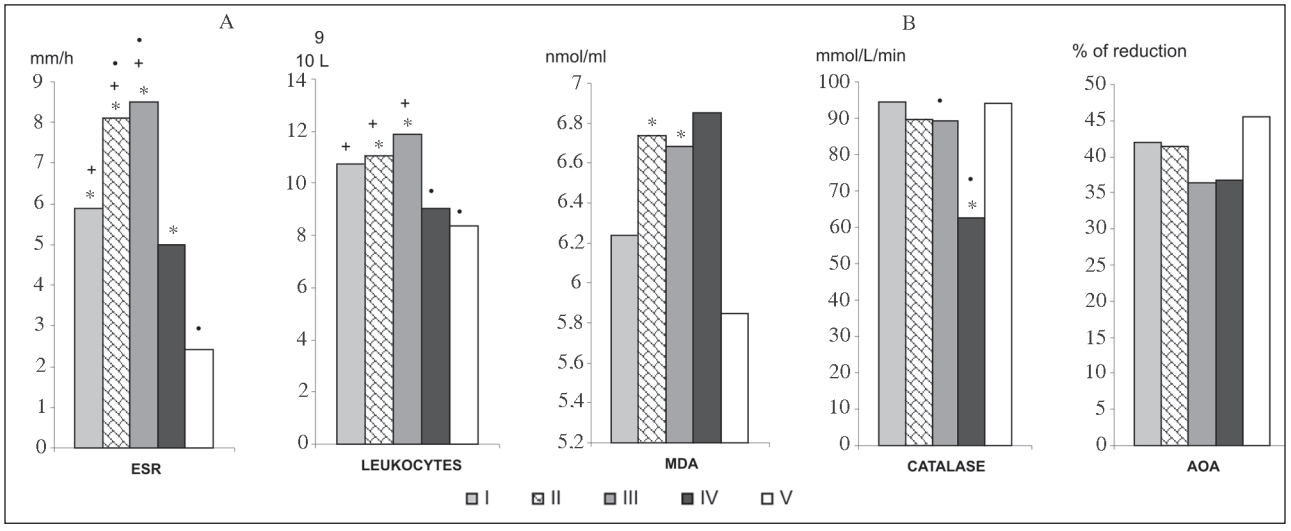

Fig. 1. Blood (**A**) and pro-/antioxidant system indices **(B)** of arthritic rats immunized with influenza vaccine. Adjuvant arthritis (AA) was induced in 30 rats via the foot pad method by injection of 0.1 ml of CFA into the left hind paw under light anaesthesia. Group I (control) – animals with induced adjuvant arthritis (AA); second two groups – rats with AA immunized intramuscularly (i. m.) on days 1, 8, 15 and 30 (prophylactic vaccination; group II) and on days 8, 15, 21 and 30 (therapeutic vaccination; group III) with 0.01 ml of single human dose (SHD) of vaccine (total 0.04 ml of SHD). Group IV – healthy animals immunized with influenza vaccine on days 1, 8, 15 and 30. Group V – non-vaccinated healthy animals. MDA – malondialdehyde, AOA – antioxidant activity. The differences are significant in comparison with healthy non-vaccinated (*) and vaccinated (+) animals and with the control group (•).

was higher in AA groups ($P < 0.02–0.01$) and lower in the healthy vaccinated group ($P < 0.002–0.001$). Groups vaccinated according to different protocols (prophylactic and therapeutic) did not differ significantly.

Changes in the blood indices are shown in Fig. 1A. The ESR and leukocyte count for all groups of rats with AA (control and vaccinated) was markedly higher ($P < 0.05–0.001$) than in the group of healthy animals. The same tendency was observed in the group of healthy vaccinated animals in which the ESR was also increased ($P < 0.01$). The erythrocyte count was the same in all groups ($5–5.5 \times 10^{12}$ L, data not shown).

Pro-/antioxidant activity of the blood serum of AA rats immunized with influenza vaccine

Pro-/antioxidant activity of the blood serum of rats is shown in Fig. 1B. Free radical formation resulting in lipid peroxidation, measured as the MDA level, was found to be elevated in all AA groups and in healthy vaccinated animals as compared with the healthy control. However, a significant in-

crease of MDA was observed in both AA vaccinated groups ($P < 0.05–0.02$) independently of the treatment protocol.

A decrease of CAT activity in the blood serum of healthy vaccinated animals in comparison with both non-vaccinated (healthy and arthritic) control groups ($P < 0.01$) was revealed. The same tendency was observed after therapeutic vaccination when CAT activity was lower than in the control AA group ($P < 0.05$).

The serum AOA levels were somewhat lower in the test groups than in the healthy animals, although the difference was not significant.

The effect of vaccination on the liver

Pathomorphological examination of the liver (Fig. 2) showed a significant increase of parenchyma alteration, inflammatory infiltration of hepatic stoma, penetration of inflammatory cells into the lobules in vaccinated rats with AA as compared with healthy vaccinated animals. V. centralis hypervolemia was higher after therapeutic vaccination ($P < 0.03$). A significant increase of lymphocyte infiltration ($P < 0.002–0.001$), a rise of plasma cells (20% of animals after therapeutic vaccination) and macrophages (30% of rats after prophylactic and 10% after therapeutic vaccination) were observed, whereas no plasma cells and macrophages were revealed in healthy vaccinated animals and rats of the control AA group. Both protocols of vaccination exacerbated necrotic processes in the parenchyma of arthritic rats ($P < 0.02$ after prophylactic and $P < 0.05$ after therapeutic vaccination) in comparison with AA control (Fig. 2A). General inflammatory infiltration increased after therapeutic vaccination ($P < 0.05$). Fibrotic changes in the liver did not differ significantly, but were more pronounced in therapeutically vaccinated arthritic animals and healthy vaccinated rats (Fig. 2B).

The effect of vaccination on the lungs

Histological examination revealed that at the end of experiment 100% of arthritic animals (vaccinated and control) had more or less pronounced peribronchiolitis, interstitial and alveolar lesions (Fig. 3). The lungs of rats with AA were similarly affected by using both protocols of vaccination. The alveoli, interstitial septa and perivascular spaces were infiltrated by a mixture of inflammatory cells. Changes were observed also in the lungs of healthy vaccinated animals, but they showed a less lung involvement – a less pronounced increase in the cellularity of the interstitial septa and a lower inflammatory infiltration of the alveoli and perivascular spaces.

Compared to healthy vaccinated animals, peribronchiolitis with a significant lymphocyte infiltration (Fig. 3A [1]) in AA control and prophylactically vaccinated rats with AA was observed ($P < 0.03$; $P < 0.02$). Peribronchial fibrosis arose in the groups of animals (healthy and arthritic) that

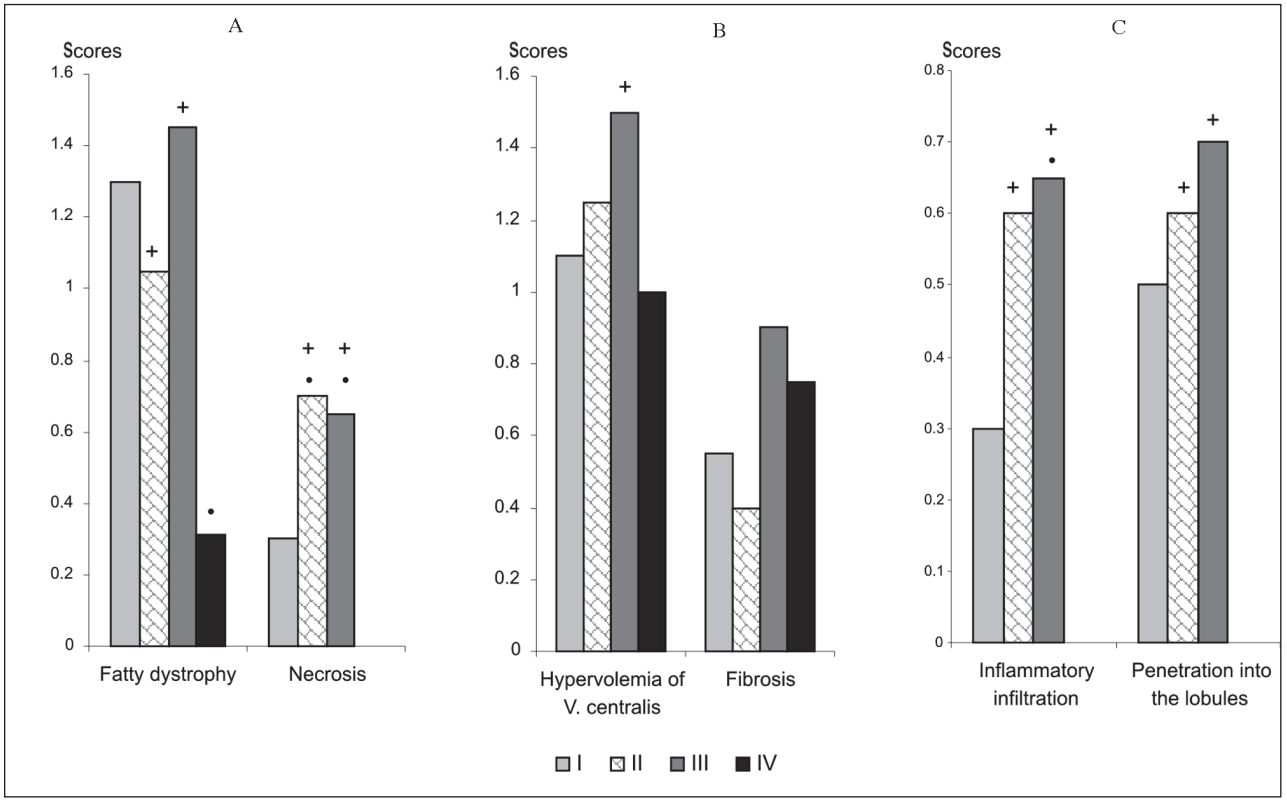

Fig. 2. Patomorphological changes in the liver of arthritic rats immunized with influenza vaccine. Group I (control) – animals with induced adjuvant arthritis (AA); second two groups – rats with AA immunized intramuscularly on days 1, 8, 15, and 30 (prophylactic vaccination; group II) and on days 8, 15, 21, and 30 (therapeutic vaccination; group III) with 0.01 ml of single human dose (SHD) of vaccine (total 0.04 ml of SHD). Group IV – healthy animals immunized with influenza vaccine on days 1, 8, 15, and 30. The differences are significant in comparison with healthy vaccinated (+) animals and with the control group (•).

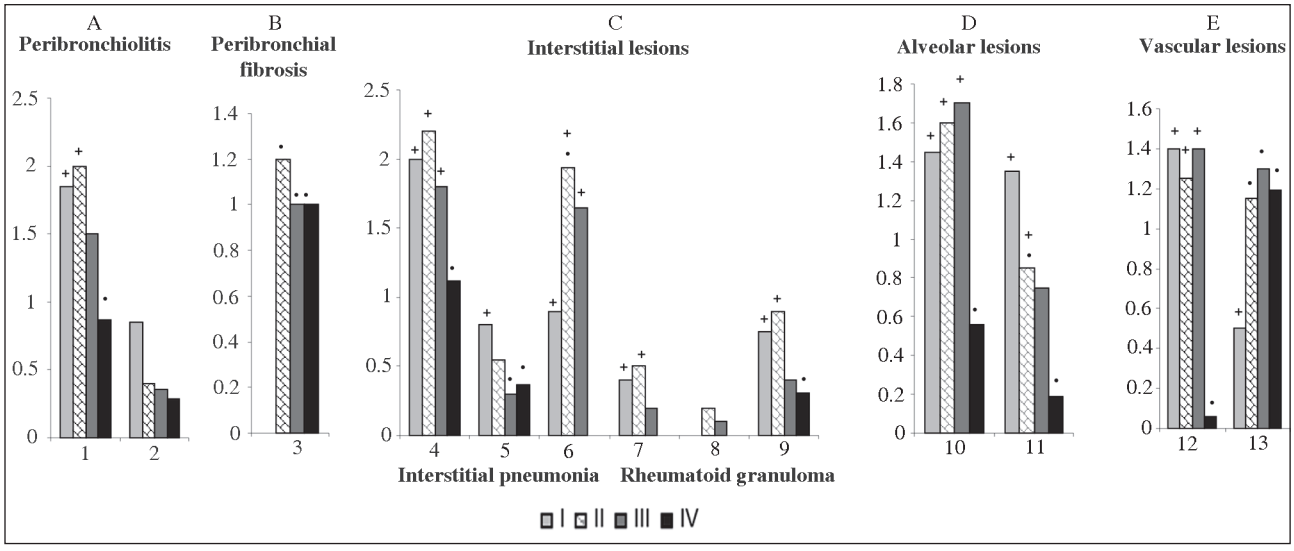

Fig. 3. Pathomorphological changes in the lungs of arthritic rats immunized with influenza vaccine Group I (control) – animals with induced adjuvant arthritis (AA); second two groups – rats with AA immunized intramuscularly on days 1, 8, 15, and 30 (prophylactic vaccination; group II) and on days 8, 15, 21, and 30 (therapeutic vaccination; group III) with 0.01 ml of single human dose (SHD) of vaccine (total 0.04 ml of SHD). Group IV – healthy animals immunized with influenza vaccine on days 1, 8, 15, and 30. A – peribronchiolitis (brochocentral granuloma) [infiltration with lymphocytes (1) and plasma cells (2)]. B – peribronchial fibrosis (3). C – interstitial pneumonia [infiltration with lymphocytes (4), plasma cells (5)] and rheumatoid granuloma [infiltration with lymphocytes (6), plasma cells (7)), giant macrophages (8), fibrosis (9)]. D – alveolar lesions [hemorrhages (10), inflammatory infiltration (11)]. E – vascular lesions [fibrinoid necrosis (12), sclerosis (13)]. The differences are significant in comparison with healthy vaccinated (+) animals. The differences are significant in comparison with the control group (•).

had received influenza vaccine ($P < 0.002$; $P < 0.008$), what significantly differed from the control AA group where it was absent (Fig. 3B); 10% of therapeutically vaccinated AA animals had obliterative bronchiolitis and 50% minimal (1+; 10%) and moderate (2+; 40%) peribronchial fibrosis.

Interstitial pneumonia (Fig. 3C [4]) with lymphocyte infiltration was the same in all AA groups (vaccinated and control) and significantly differed only from the healthy vaccinated group of animals ($P < 0.002–0.001$), whereas the count of plasma cells was markedly lower in healthy ($P < 0.01$) and therapeutically vaccinated arthritic animals ($P < 0.02$) as compared with the control AA rats (Fig. 3C [5]).

Interstitial lesions of vaccinated and non-vaccinated arthritic animals were accompanied by a rise of rheumatoid granuloma. Vaccinated rats with AA exhibited a severe inflammation. An increase of lymphocytes, plasma cells, and the rise of giant macrophages was observed. A two-fold and 1.8-fold increase of lymphocyte infiltration was observed in both AA vaccinated groups (Fig. 3C [6]), although significant differences in comparison with the control group were revealed only for the group prophylactically treated with influenza vaccine ($P < 0.03$). Giant macrophages (Fig. 3C [8]) appeared in prophylactically (20% of rats) and therapeutically (10%) vaccinated rats. No cases of rheumatoid granuloma in the healthy vaccinated animals were found.

Fibrotic processes were more pronounced in arthritic than in healthy vaccinated animals, but the intensification was significant only in the prophylactically vaccinated group ($P < 0.04$). Fibrosis in this group was by 20% higher than in the control group (Fig. 3C [9]).

Alveolar lesions in non-vaccinated and vaccinated groups of arthritic animals were accompanied by a rise of hemorrahages and inflammatory infiltration (Fig. 3D). These changes significantly differed from changes in the group of healthy vaccinated animals ($P < 0.004–0.0001$). Although there were no significant differences in the prophylactically and the therapeutically vaccinated groups as compared to AA control, cases of hemorrahages in the vaccinated groups were more frequent (by 10.3% and 17.2%) (Fig. 3D [10]). Inflammatory infiltration was most pronounced in the control AA group (Fig. 3D [11]), and its significant decrease was revealed in the prophylactically vaccinated group ($P < 0.02$).

Investigation of vascular lesions showed fibrinoid necrosis, perivascular fibrosis and sclerosis in the lungs (Fig. 3E). Fibrinoid necrosis was the same in all arthritic groups (Fig. 3E [12]) and significantly differed from that in the healthy vaccinated group ($P < 0.001$). It should be noted that vaccination intensified sclerotic processes in arthritic rats ($P < 0.02$) and healthy animals ($P < 0.001$) as compared with control (Fig. 3E [12]). Of the therapeutically vaccinated animals, 10% had obliterative endovasculitis and 40% had minimal (1+; 10%) and moderate (2+; 30%) perivascular fibrosis.

Joint swelling in adjuvant-injected paw and polyarthritis development

Development of inflammation was checked by measuring plethysmographically the volume of adjuvant-injected paws three times per week. Figure 4 shows the results of an experiment in which two groups of AA rats were immunized with influenza vaccine i.m. four times. The vaccination was associated with a significant worsening of the disease. As com-

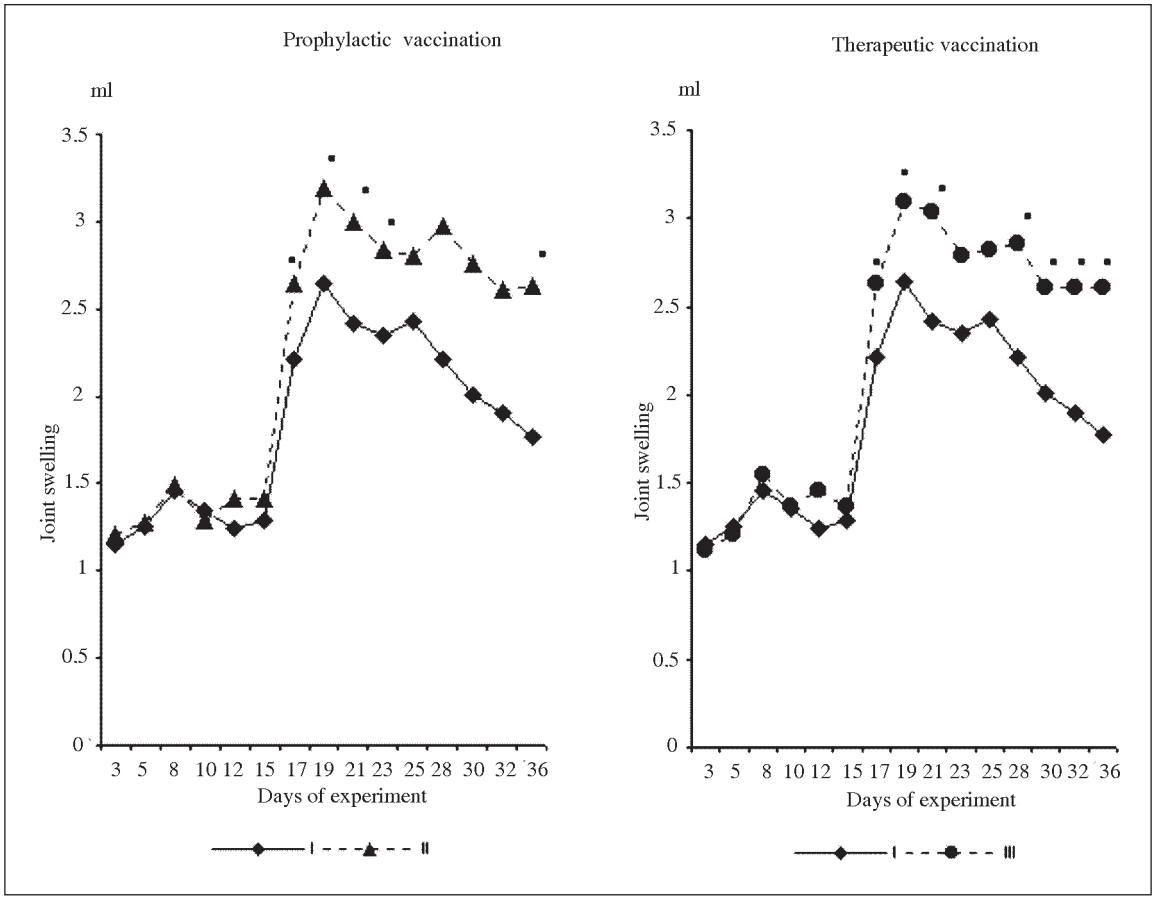

Fig. 4. Joint swelling in arthritic rats immunized with influenza vaccine. Group I (control) – animals with induced AA; second two groups – rats with AA immunized intramuscularly on days 1, 8, 15, and 30 in prophylactic vaccination (group II) and on days 8, 15, 21, and 30 in therapeutic vaccination (group III) with 0.01 ml of single human dose (SHD) of vaccine (total 0.04 ml of SHD). The differences are significant in comparison with the control group (•).

pared with the AA control group, both vaccinated groups showed a significant increase in joint swelling since day 17. The significance in the prophylactically treated group was observed during 17–23 days ($P < 0.05–0.01$) and on day 35 ($P < 0.05$) and in the therapeutically treated group during days 17–21 ($P < 0.02–0.01$) and 28–35 ($P < 0.05–0.002$). Although the groups vaccinated by different protocols did not differ with regard to joint swelling, the significance in comparison with the control group was higher when the therapeutic protocol of vaccination had been used.

Development of polyarthritis, characterizing the generalization of the disease and exacerbation of the autoimmune process, was the same in all test groups, although in vaccinated groups it was more pronounced and joint swelling was observed in all four limbs.

Throughout the course of vaccination, no clinical signs of joint swelling or effects of the autoimmune process were apparent in healthy animals.

Histopathology of joints

Histological examination of soft tissues surrounding the ankle joints in all arthritic groups (control and vaccinated) showed a pronounced inflammatory infiltration with lymphocytes, leukocytes, macrophages, edema, angiomatosis and connective tissue disorganization (Table 2). Both prophylactic and therapeutic vaccination enhanced infiltration with leukocytes (by 42% and 64.5%; $P < 0.03$; $P < 0.002$) and macrophages (by 7.7% and 19.2%) in both groups of arthritic animals. Edema (difference is close to significant in comparison with the control group; $P < 0.056$) and angiomatosis ($P < 0.007$) increased respectively by 22.2% and 58.3% in the therapeutically vaccinated group. Thus, the therapeutic use of the vaccine induced more pronounced changes in soft periarticular tissues. It should be noted that slight signs of edema and fibrosis were also found in one healthy vaccinated rat.

Synovium villi proliferation, edema, γ-metachromasia, inflammatory infiltration with lymphocytes, granulocytes, macrophages, fibrotic processes and angiomatosis significantly increased in all AA groups versus healthy vaccinated rats (Table 2). However, no significant differences between the AA-vaccinated groups and control were found, although an increase in synovium villi proliferation (by 63%), edema (by 13%), infiltration with granulocytes (by 44.4%), and macrophages (by 55.6%) was observed in the therapeutically vaccinated group. Prophylactic vaccination enhanced infiltration with macrophages by 33.3%.

Table 2. **Pathomorphological changes in joints of healthy and arthritic rats immunized with influenza vaccine**

Index			Groups			
			I (AA control) n = 10	II (AA + prophylactic vaccination) n = 10	III (AA + therapeutic vaccination) n = 10	IV (Healthy vaccinated animals) n = 10
Soft periarticular tissues	Inflammatory infiltration	Lymphocytes	+1.90 ± 0.14	+1.95 ± 0.12	+1.85 ± 0.08	–•
		Plasma cells	0.05 ± 0.05	0.05 ± 0.05	0.05 ± 0.05	–
		Leukocytes	+1.55 ± 0.20	+2.20 ± 0.15•	+2.55 ± 0.12•	–•
		Macrophages	+1.30 ± 0.15	+1.40 ± 0.12	+1.55 ± 0.22	–•
		General	+2.30 ± 0.08	+2.45 ± 0.09	+2.50 ± 0.10	0.14 ± 0.14•
	Edema		+1.80 ± 0.13	+1.60 ± 0.12	+2.20 ± 0.13	–•
	Angiomatosis		+1.20 ± 0.18	+1.45± 0.14	+1.90 ± 0.12•	–•
	Fibrosis		0.50 ± 0.27	0.45 ± 0.24	0.20 ± 0.13	0.07 ± 0.07
	Disorganization of connective tissue (γ-metachromasia)		+0.72 ± 0.17	+0.90±0.12	+1.00±0.18	–•
Synovium	Villi proliferation		+1.60 ± 0.10	+1.60 ± 0.16	+1.70 ± 0.13	0.44 ± 0.15•
	Edema		+1.15 ± 0.22	+1.15 ± 0.24	+1.30 ± 0.20	0.06 ± 0.06•
	Disorganization of connective tissue (γ-metachromasia)		+0.55 ± 0.16	+0.55 ± 0.11	+0.45 ± 0.14	–•
	Inflammatory infiltration	Lymphocytes	+1.45 ± 0.12	+1.50 ± 0.18	+1.45 ± 0.16	–•
		Plasma cells	0.10 ± 0.07	0.10 ± 0.07	0.05 ± 0.05	–
		Granulocytes	0.45 ± 0.20	+0.40 ± 0.12	+0.65 ± 0.13	0.06 ± 0.06
		Macrophages	+0.45 ± 0.16	+0.60 ± 0.12	+0.70 ± 0.13	–•
		General	+1.55 ± 0.12	+1.50 ± 0.18	+1.60 ± 0.10	0.06 ± 0.06•
	Fibrosis		0.20 ± 0.13	0.25 ± 0.13	0.25 ± 0.13	–
	Angiomatosis		+1.65 ± 0.21	+1.65 ± 0.15	+1.55 ± 0.26	0.06 ± 0.06•
Cartilage	Erosium		+1.80 ± 0.13	+1.90 ± 0.27	+2.05 ± 0.05	0.31 ± 0.13•
	Usure		+0.95 ± 0.23	+0.90 ± 0.23	+1.25 ± 0.18	–•
	Fissure		–	0.05 ± 0.05	0.30 ± 0.15	–
	Acid glycosaminoglycans		+2.10 ± 0.19	+1.95 ± 0.27	+1.80 ± 0.11	3.00 ± 0•
	Pannus		+1.60 ± 0.18	+1.25± 0.20	+1.35 ± 0.17	–•
	Thinning of cartilage		+0.60 ± 0.18	+0.60 ± 0.23	+0.75 ± 0.25	–•

Note. Adjuvant arthritis (AA) was induced in 30 rats by the foot pad method by injecting 0.1 ml of CFA into the left hind paw under light anaesthesia. Group I (control) – animals with induced AA. Second two groups – rats with AA immunized intramuscularly (i. m.) four times on days 1, 8, 15, and 30 in prophylactic vaccination (group II) and on days 8, 15, 21, and 30 in therapeutic vaccination (group III) with 0.01 ml of single human dose (SHD) of vaccine (total 0.04 ml of SHD). Group IV – 8 healthy animals immunized with influenza vaccine on days 1, 8, 15, and 30. The differences are significant in comparison with healthy vaccinated (+) animals and with the control group (•).

Significant changes in the cartilage were found only in comparison with healthy vaccinated animals. Although the obtained changes did not differ from those in the control AA group, therapeutic vaccination enhanced erosium and usures by 13.9% and 31.6%, respectively. Superficial fissures were induced in 10% of animals by prophylactic vaccination, and moderate fissures were found in 30% of rats after therapeutic vaccination. No signs of fissures in the control AA group were observed. Both after prophylactic and therapeutic vaccination, glycosaminoglycans decreased respectively by 7.2% and 14.3%. Cartilage thinning was higher by 25% after therapeutic vaccination. Thus, therapeutic vaccination in the case of an established pathological process induces more pronounced changes in the joints.

Although we failed to detect any evidence of a clinically observed autoimmune process in healthy rats after vaccination, it should be noted that traces and minimal changes of synovial proliferation (respectively in 37.5% and 25% of animals) and cartilage erosium (in 37.5% traces and in 12.5% minimal changes) were observed. Edema, inflammatory infiltration with granulocytes, and angiomatosis in the synovium were found in one rat (12.5%).

DISCUSSION

Concerns about the safety and efficacy of immunizing subjects with connective tissue diseases have persisted for over 50 years (28, 29). Such studies have become important because serious problems have arisen with the use of some killed vaccines.

Studies in laboratory animals with autoimmune disease may help to advance our knowledge of vaccines. Despite the fact that influenza vaccine has never been used for vaccination of animals with autoimmune disease, it is highly possible that it could worsen the course of the pathological process. Vaccine components such as adjuvant (e. g., aluminum), stabiliz-

ers (e. g., gelatin), preservatives (e. g., thimerosal) and residual yeast proteins from cell cultures (e. g., fibronectin) might trigger the induction of autoimmunity (5, 12, 30). It is known that thimerosal in genetically susceptible mice (A.SW mice) induces a systemic autoimmune syndrome very similar to that seen after treatment with inorganic mercury, although a higher absorbed dose of Hg is needed while using thimerosal (31).

Viral superantigens can also activate T cells through the variable domain of the TCR-β chain (32) and may therefore contribute to the established autoimmune process and induce relapses and exacerbation of the disease.

It is possible that vaccination against infectious diseases activates pathways of molecular mimicry in genetically susceptible hosts, and this may be the basis of adverse reactions to vaccines (33). Although, no data convincingly demonstrate that mimicry is an important mechanism in the development of autoimmune disease in humans.

Vaccination may produce interferon-gamma or other inflammatory cytokines in the target organ, which in turn induce human leukocyte antigen class II expression, for the first time, in non-immune cells (3). This can lead to presentation of autoantigens and activation of autoreactive T cells (34). It is a known fact that the early induction of IFN-gamma may reflect the presence of activated natural killer cells, while the later response may be the product of antigen-specific T cells (35).

Using AA, a model of RA, we have demonstrated that vaccination can enhance the phenomenon of autoimmunity. To our knowledge, this study is the first aimed to determine the response of arthritic rats to influenza vaccine. We explored inflammatory indices, biochemical changes, clinical observation data and histopathology to monitor the autoimmune disease and assess the effect of vaccination on the development of the autoimmune process. Additionally, we had an opportunity to compare not only joint lesions in both groups of vaccinated arthritic rats and healthy animals, but also to investigate the histology of the internal organs such as liver and lungs. So far, it is unknown whether inflammation or other potential pathologic changes occur in the periarticular tissues, synovium and cartilage of vaccinated rats. We therefore carried out a detailed histological analysis of joint samples obtained from healthy and arthritic vaccinated rats. Our preliminary findings revealed a certain relationship between vaccination and the exacerbation of autoimmune disease. Four i.m. administrations of the vaccine resulted in a significant increase of joint swelling, development of a more pronounced autoimmune process and histological changes. Furthermore, the route of vaccination (prophylactic or therapeutic) had no essential effect. The course of the disease was similar in both treated groups. The observed joint swelling was consistent with the histological profile of changes in soft periarticular tissues (infiltration of inflammatory cells, edema and angiomatosis) following immunization. Although, as compared to control, there were no significant synovium and cartilage changes associated with i.m. vaccination, they were more obvious in the AA vaccinated groups. An interesting observation resulting from this analysis was that therapeutic vaccination in the case of the established pathological process induced more changes in the joints, although these changes between the vaccinated groups were not significant.

By contrast, a comparatively weak response to vaccination was observed in healthy animals. No clinical signs of joint swelling and effects of the autoimmune process were apparent in these rats. We found no evidence of significant inflammation or other histological abnormalities, except only slight signs of edema and fibrosis in soft periarticular tissues, inflammatory infiltration with granulocytes, edema and angiomatosis in the synovium of one rat and traces and minimal changes of synovium proliferation and erosium of cartilage in some of these vaccinated animals. These findings suggest that influenza vaccine may not affect the joints of healthy animals.

Numerous studies have shown that in the course of AA not only joints with obvious signs of inflammation, but also visceral organs may be affected by the pathological process. Changes in protein metabolism (36) implicate hepatic involvement in the adjuvant disease, and the weight of the liver increases. It is also known that body and thymus weight is reduced significantly in arthritic rats as compared to non-arthritic animals (37). As documented elsewhere (38–40), one diagnostic characteristic of systemic inflammation in experimental arthritis is a gradual increase in spleen weight, and splenomegaly is used as a pathological index of AA. Vaccination worsened the systemic parameters of the disease, such as blood indices and organs' weight. Both the absolute and the relative weight of the spleen was the highest in the vaccinated groups with AA.

Our results obtained in arthritic rats suggest that the administration of influenza vaccine not only exacerbated the autoimmune process, but also induced marked histological changes in the internal organs. Both protocols of vaccination significantly exacerbated necrotic processes in the liver parenchyma of arthritic rats. In response to vaccination, significant changes in the lungs were observed. Peribronchial fibrosis and interstitial damage with inflammatory lymphocyte infiltration were evident in lung sections of influenza vaccine-immunized animals. Vaccination significantly intensified vascular sclerosis in arthritic rats and healthy animals. It has been shown that a lesion of respiratory epithelium and the subsequent activation of monocytes / macrophages results in a release of proinflammatory cytokines (TNF-α, IL-6). Influenza A virus pneumonia in mice is known to induce the production of cytokines and chemokines in lung tissues (41–43).

Oxidative stress plays a key role in the inflammation and destruction of RA and animal arthritis joints (44, 45). Our previous studies (46) have shown that AA changes the response of the oxidative system in rats. One of the indices of oxidative damage is the formation of MDA as the end

product of lipid peroxidation (47). We found that MDA activity in blood serum was elevated in vaccinated animals (independently of treatment protocol) which subsequently developed more marked arthritic signs and had histopathological lesions in joints. Inflammatory cells promote injury through a combined action of free radicals and cytokines. Macrophages have also been shown to be capable of promoting joint damage *in vivo* and *in vitro* through a release of free radicals. Enhanced infiltration with macrophages, especially after therapeutic vaccination, was observed in our study.

Therapeutic vaccination of arthritic rats significantly decreased CAT activity in their blood serum. The observed changes in serum AOA activity were similar in both vaccinated groups and did not differ from the AA control.

Although healthy vaccinated animals showed no clinical symptoms of autoimmune disease or had minimal histopathological lesions in joints, there were several biochemical and hematological changes (an increase of ESR, MDA and a decrease of CAT activity) in these animals, which were followed after vaccination.

The relationship between the severity of the disease and vaccination is not yet clear. Nevertheless, we believe that the pathogenesis of the vaccine-enhanced AA disease is complex and multifactorial, and its comprehensive understanding will require studies of chemical mediators (cytokines and chemokines) as well as histopathological investigations. Besides, the histopathological studies described in the current report are basic rather than complete, and the detection of cell-surface markers in the vaccinated rats, particularly of T lymphocytes, will shed additional light.

CONCLUSIONS

Although we failed to detect any evidence of a clinically observed autoimmune process in healthy rats after vaccination, except only minimal histological changes in joints of some animals, our findings imply two main conclusions.

First, vaccines can generate and support a more pronounced inflammation when injected to animals with autoimmune disease. It was confirmed by increased joint swelling, pathomorphological changes in joints and internal organs, increased MDA production. Therapeutic vaccination in case of an established pathological process induces more pronounced changes in joints.

Second, further studies are needed to support our findings and to explore the influence of vaccines on the autoimmune process.

We believe that our investigations warrant the further research since a better understanding of the pattern leading to the autoimmune phenomenon following vaccination will trigger great progress in the field of vaccination and autoimmunity.

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GRIPO VAKCINA IMUNIZUOTŲ ŽIURKIŲ AUTOIMUNINIO PROCESO EIGA

Santrauka

Prielaidos ir tikslas. Nors vakcinacijos vaidmuo autoimuninėms ligoms gana aktyviai tyrinėjamas, tačiau šiuo klausimu kyla daug diskusijų. Dar reikia įrodyti, ar egzistuoja priežastinis ryšys tarp vakcinacijos ir autoimuninių reiškinių atsiradimo. Tam reikalingi tyrimai su gyvūnais. Šio darbo tikslas – įvertinti Wistar žiurkių, imunizuotų gripo vakcina, autoimuninio proceso eigą.

Medžiaga ir metodai. Darbui panaudoti 43 Wistar veislės žiurkių patinai: 30 buvo sukeltas adjuvantinis artritas (AA), 13 buvo sveiki. Dvi grupės gyvūnų su AA ir viena sveikų žiurkių grupė buvo keturis kartus imunizuotos 0,01 ml žmogaus A gripo vakcina. Atlikta profilaktinė ir terapinė vakcinacija suleidžiant preparatą į raumenis. Kontrolines grupes sudarė nevakcinuoti gyvūnai su AA ir sveiki. Įvertinta kūno ir organų masė, kraujo ir pro / antioksidantinės sistemos rodikliai serume, sąnarių patinimas, poliartrito atsiradimas ir histologiniai pokyčiai sąnariuose, kepenyse ir plaučiuose.

Rezultatai. Vakcinuotiems gyvūnams buvo stebimas agresyvesnis patologinis procesas: padidėjo ENG ir malondialdehido (MDA) kiekis, atsirado histologinių pokyčių plaučiuose ir kepenyse. Nepaisant vakcinuotų gyvūnų su AA statistiškai reikšmingo sąnarių patinimo ir didesnių pokyčių minkštuose audiniuose, dengiančiuose sąnarį (uždegiminės infiltracijos, edemos ir angiomatozės), pokyčiai sinovijoje ir kremzlėje nesiskyrė nuo AA kontrolės. Terapinė vakcinacija sukėlė didesnę edemą ir ryškesnę granuliocitų ir makrofagų uždegiminę infiltraciją sinovijoje, fisuras 30% gyvūnų, sustiprino erozijų (13,9%) ir uzurų (31,6%) atsiradimą bei kremzlės suplonėjimą (25%). Sveikiems gyvūnams po vakcinacijos nerasta autoimuninio proceso požymių, išskyrus nedidelius histologinius pokyčius keleto žiurkių sąnariuose.

Išvados. Mūsų preliminariais duomenimis, komercinės gripo vakcinos panaudojimas gali sustiprinti gyvūnų autoimuninį procesą, lemiantį didesnį sąnarių patinimą, histologinius pokyčius minkštuosiuose sąnario audiniuose bei plaučiuose. Terapinės vakcinacijos poveikis buvo ryškesnis.

Raktažodžiai: žiurkės, adjuvantinis artritas, vakcinacija