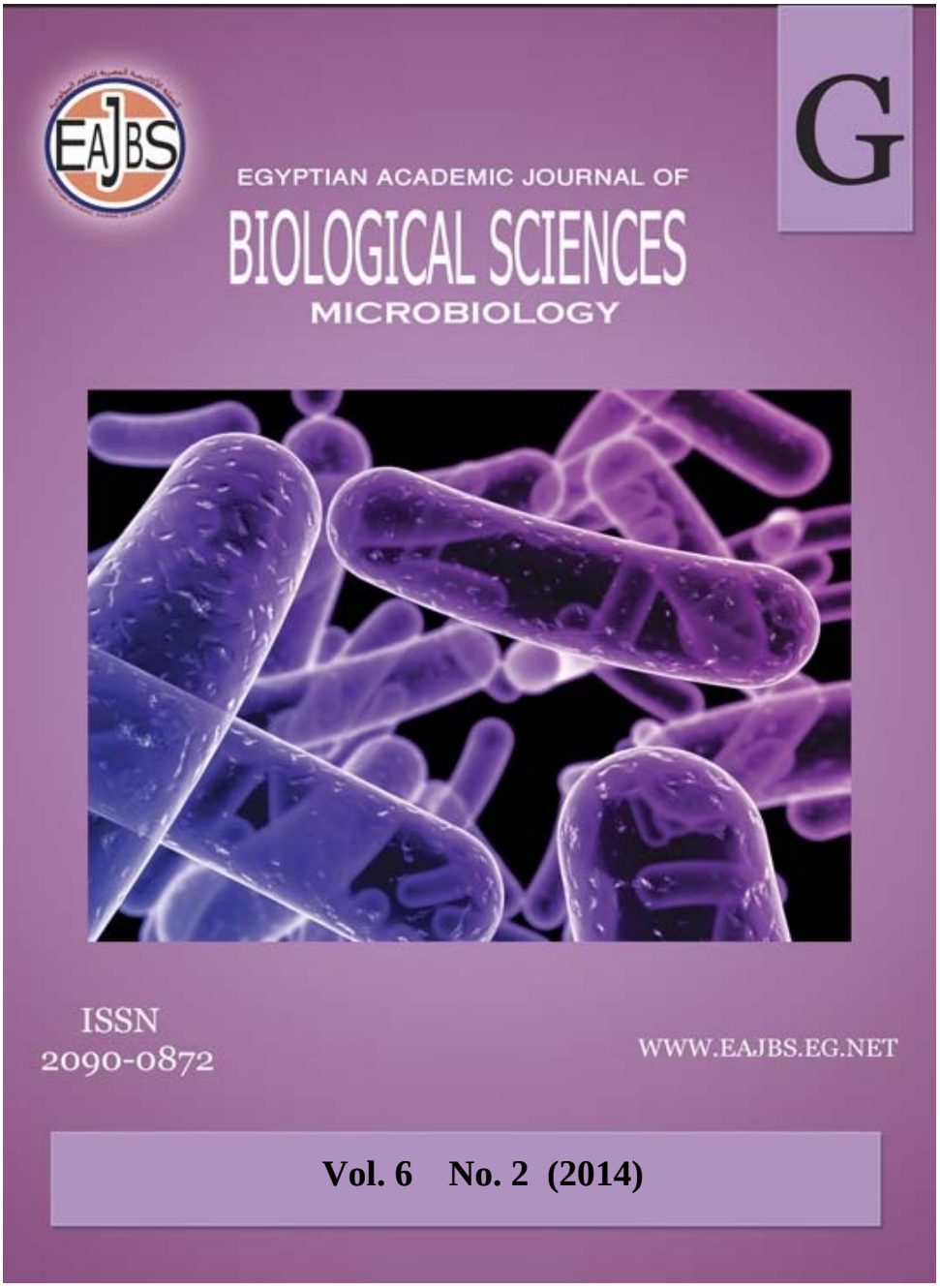

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Effects of Allergic fungi on hematological and immunological parameters of human patients and rabbits

Sanaa Huseein Mohamad[1]; Karkaz M. Thalij[2]; Khalil AL-Bander[3] and Batol Imran Dheeb[4]

1- Biology Department, College of Science, Kirkuk University, Iraq
2- Food Science Department, College of Agriculture, Tikreet University, Iraq
3- Biology Department, College of Science, Tikreet University, Iraq
4- Biology Department, College of Science, Iraqia University, Iraq.

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ABSTRACT

The hematological parameters and immunoglobulin levels were estimated in patients and healthy controls as well as experimentally infected lab animals. Rabbits were infected with fungi (*A. niger, C. albicans, A. fumigatus, A. flavus, Fusarium* spp, *Alternaria* spp). For 21 days before serum collection. Two hundred serum samples were collected from the respiratory tract infected patients and 20 samples from age and sex matched healthy persons. Hematological parameters in human and rabbits indicated high significance ($p<0.001$) of most blood parameters compared with control groups including leukocytosis; lymphocytosis; eosinophilia and increased ESR. ELISA testsshowed highly significant levels of IgE and IL4 ($P\leq0.001$) in patients compared with the healthy people, and the highest increased were with the fungus *Aspergillus fumigatus* (450 IU /ml; 15pg / ml). Similarly, the levels of IgE and IgM in rabbits inhaled with fungi had significantly increased to 425 IU /ml, 390 mg/dl with *Aspergillus fumigatus*.

INTRODUCTION

The term allergy was coined in 1906 by a Viennese pediatrician named *Clemenson Pirquet* to describe a hypersensitive immune reaction in response to a substance other than a typical disease causing agent (Wagner, 1968). However, the word “allergy” is derived from the Greek words *allos* which means "other" and *ergon*means "reaction" or "reactivity." The word allergy is most commonly used in reference to type-I, or to immediate onset of hyper sensitivity which is characterized as an inflammatory reaction caused by excessive activation of IgE bound mast cells as response to a specific but typically benign antigen. The most common clinical allergy symptoms "hay fever", includes runny nose, itchy eyes and sneezing; however severe allergic reactions can lead to anaphylactic shock and even death (Kurup and Banerjee, 2000;Gould *et al*., 2003).

The type-I hypersensitivity, nowadays, is a growing problem in the society. Allergic disease is projected to affect 20-25% of the population of the world's industrialized nations out of those, 10% develop severe allergic disease (Horner *et al.,* 1995; Kurup *et al.,* 2002).

The "National Institute of Allergy and Infectious Diseases" estimate that about 50 million Americans are affected by allergic diseases in the United States alone, with allergies constituting the sixth leading cause of chronic disease (Sagi-Eisenberg, 2002).

The aims of the study were to determine the effects of allergic fungi in hematological and immunological parameters.

MATERIAL AND METHODS

Blood Samples

Blood samples were collected from patients (10 mL), (2.5 mL) inside anticoagulant tube for hematological test while another 7.5 mL was laced in plain tube without anticoagulant material. Serum was separated with centrifuge and placed in sterile plastic tubes and kept at (-20°C) in Public Health Laboratory at Kirkuk province upon request.

And also blood samle collected from Rabbit to study Effects of (*A. niger, C. albicans, A. fumigatus, A. flavus, Fusarium*spp, *Alternaria*spp) spore suspention. Inhalation of fungal conidia on IgE and IgM levels in Rabbit.

Hematological parameters

This machine was used for hematological test; the principle depends upon the automatic multi-parameter, blood cell counter by using multi-wave length. White blood cells (WBC), red blood corpuscles (RBC) and platelets were counted using the direct current detection method with coincidence correction. Automatic discriminators separate the cell populations based on complex algorithms. The intensity of the electronic pulse from each analyzed cell is proportional to the cell volume. The hematocrit (HCT) does directly determine the red cell count and volume of each individual RBC. Even with samples at extremely low or unusually high concentrations, the system cell counter does analyze the WBCs, RBCs and PLTs with uncompromised precision and accuracy.

Immunological tests:

Total immunoglobulin E (IgE) levels in sera.

Determination of IgE levels in was done according to (Feldmesser*et al*. (2007) using ELISA kit (name of manufacturer) according to manufacturer instructions. Samples from patients as well as healthy controls were used. The absorbance of every well was read at 450 mm in a micro-plate reader (winti, Italy).

Determination of the IgM antibodies levels

Determination of IgM levels was done according to Ibrahim. (2000) using radial immune diffusion kit (name of company) according manufacturer's instructions.

The examined protein, diffused in agarose gel contained a specific antibody did form an immune-complex; visible as a ring around the well. The ring diameter is direct proportion to the concentration of the analyzed protein. The proportion corresponds to the diffusion time in fact, at the end (96 hr), the square of the diameter will be in linear proportion to the concentration of the sample. With the Plate is supplied a reference tube in which each diameter of the halo is associated and concentrated.

Procedure:

The plates were removed from their envelope and were left to stand at room temperature for few minutes to have any condensed water in the wells be evaporated. The wells were filled with 5 mL of each sample and/or controls and waited until completely absorbed before handling the plate. The plates were closed and placed in a moist chamber for 96 hours.

Interpretation of Results:

Precipitating ring with appropriate has been measure by ruler or measuring lens with a system provides a maximum error of

0.1 mm. Enclosed reference table was measured with the concentration value corresponding the precipitating ring diameter. The control serum used should always give a ring which differs by a maximum of 0.2 mm from the value reported in the tube.

Human IL-4-ELISA

The Omikin^{Tm} Human IL-4-ELISA kit contains the components necessary for quantitive determination of the natural HIL-4 concentrations within experimental sample including cell Lysates, serum and plasma was used.

The (Omnokine^{TM}) procedure.

1. Reconstitute Biokin-Conjugate detection Antibody and protein standard and dilute the 10Xwash buffer as specified.
2. Perform serial dilution of protein standard and primer samples desired.
3. Add 100 ml of standard, sample or control to each well and incubate for 2 hours at room temperature.
4. Aspirate standard samples or control out and wash plate 4 times.
5. Dilute Biotin – conjugated detection antibody as specified. Add 100 ml to each well of incubate for 2 hours at room temperature.
6. Aspirate Biotin – conjugate detection Antibody of wash plate 4 times.
7. Add 100 ml of ready-to-use streptavidin – HRP to each well and incubate at room temperature for 30 minutes.
8. The ready-to-use Avidin-HRP was aspirated out and the plate was washed 4 times.
9. 100 mL of ready-to-use substrate was added to each well and incubated at room temperature.
10. 100 mL of stop solution of was added to the reading plate at 450 mm.

RESULTS AND DISCUSSION

The Hematological parameters in human patients after allergic fungal infection

Blood parameters Hb%, HCT, WBC with differential counting cells and ESR revealed significant decrease ($p \leq 0.01$) while the differential counting had significantly increased ($p \leq 0.01$) in comparison with control patients as showed in Table 1. These results are in agreement with Tung, *et al*., (1975a) and Lanza, *et al*., (1980).Others reported that aflatoxin B1 causes anemia observed as decreases PCV and Hb and increases ESR values. Ibrahim (2000) showed that Hb% and PCV values were increased in rabbits orally treated with aflatoxin B1 (15 or 30 ng /kg body weight) every other day. The Hb% and PCV decreased while total leucocytes count did not affect in rabbits treated with 15 µg /kg body weight of aflatoxin B1 orally every day for nine week (Yousefa, *et al*., 2003).

Table 1: The Hematological parameters in patients with fungal infection.

Type of infectious fungus	CBC								
	Hb (g /dl)	HCT (%)	WBC (X 10^3/mm^3)	L (%)	N. (%)	E. (%)	Mon. (%)	B. (%)	ESR (mm/hr at 25 °C)
Control	9.1b±**0.22**	28b±**0.4**	6**c**±**0.9**	38d±**0.0**	60a±**0.51**	1c±**0.19**	1**a**± **0.2**	0**b**±**0.0**	15**e**±**0.12**
C.albicans	10a± **0.4**	31**a**±**0.4**	14b±**0.7**	60b±**0.8**	33c±**0.8**	7b±**0.14**	0b± **0.0**	0**b**± **0.0**	56**d**±**0.33**
A. niger	9.5a± **0.9**	29b±**0.6**	16a±**0.5**	56c±**0.9**	36b±**0.3**	8a± **0.9**	0b±**0.0**	0**b**± **0.0**	82b±**0.51**
A. flavus	9.7a± **0.9**	29b±**0.7**	14b± **0.7**	54c±**0.9**	37b±**0.2**	7b± **0.9**	1a±**0.5**	1a±**0.12**	64**c**±**0.81**
A.fumigatus	10.3a± **0.5**	31.3a± **0.2**	17a± **0.9**	64a±**0.8**	27d±**0.1**	9a± **.10**	0b±**0.0**	0**b**±**0.0**	93a±**0.12**

Hb=Haemoglobin; **HCT**=Hematocrit;-**WBC**=Leukocytes , **L**=Lymphocytes, **N** =Neuterophils,

The primary route of human infection is *via* the inhalation of these airborne spores followed by conidial deposition in the bronchioles or alveolar spaces. In healthy individuals, those conidia that are not removed by mucociliary clearance do encounter epithelial cells or alveolar macrophages, the primary resident phagocytes of the lung. Alveolar macrophages were primarily responsible

for the phagocytosis and killing of *A. fumigates* conidia as well as the initiation of a pro-inflammatory response that recruits neutrophils (one type of polymorph nuclear cell (PMN) to the site of infection. Also *A. fumigatus* was produced some toxins, enzyme cell wall compounds, pigmentation and haemolysin (Hohl and Feldmesser, 2007). Gliotoxin inhibits phagocytosis by macrophages and can induce their apoptosis, the patients susceptible to invasive fungal infection. This toxin would play an important role in the pathogenesis of *A. fumigatus* (Orciuolo, *et al*., 2007).The toxic effect of hemolysin (Asp-HS), the protein is secreted into the host and can kill cells that are in the vicinity of the spore. In I Apatients this secrete can be found in the urine (Latgé, 1999) . The hemolysin, which enables the fungus to disrupt blood cells, contains negatively charged domains and can also be detected in infected patients (Malicev, *et al.*, 2007). Hemolysin lyses RBCs by creating pores or holes in red blood cell membranes resulting in the release of iron that promotes microbial growth (Bullen, 1981). *C.albicans* enables it to adapt to various environmental cues reflecting the host such as nutrient limitation, presence of serum, neutral or alkaline *p*H and 37 º C temperatures (Netea, *et al.,* 2006). Yeast hyphal transitions are also important for evading the host's immune system by escaping phagocytosis (Lorenz, *et al.,* 2004).

Antibody IgE and IL-4 after allergic fungal infection

Table 2 summarize all information obtained from ELISA Teqnique, this test is more effective for diagnosis of LRT infection with fungi, sputum and/or pharyngeal. Aspirates are not valid for the assessment of the extent of colonization of the LRT of cystic fibrosis (Maiz *et al.,* 2008).

Table 2: The IgE and IL-4 levels prevalence in patients with fungal infection.

Fungal Species	*IgE (I.U / ml)	**HIL-4 (pg /ml)
Control	60e±1.1	4d± 0.9
A. fumigatus	450a± 0	15a± 0.2
A. niger	330b±1.5	12b± 0.25
A. flavus	240d± 0.5	8c± 0.1
C. albicans	270c± 2.3	9c±0.12

*IgE:Immunoglobuline E, **HIL-4: Human Interleukin-4

The level of IL-4 sera in LRT patients by indirect ELISA technique which showed an insignificant increased difference to most of cases when compared with control. The production in total IL-4 levels in pulmonary function has been reported hyper increase of IL-4 with eosinophils in total serum IL-4 levels and Aspergillus IgG levels (Afshari, *et al*; 2003). The IL-4 was pleiotropic cytokines contributing to the maintenance of the Th2 lymphocyte profile that leads to the elevation of base line IgE levels. In contrast to IL-4, which is produced by peripheral blood CD4+ T cell, IL-13 is produced by CD4+ as well as CD8+ T-cells and by both naïve and memory Tcells (Howard, *et al.,* 2002).

Many factors affect the titer of antibody including: type of carrier used in coupling, concentration of antigen, type of pathogen (Maiz, *et al*., 2008). Patients with atopic allergic diseases such as atopic asthma, atopic dermatitis, and high fever have been shown to exhibit increased total immunoglobulin E (IgE) levels in blood. IgE is also known as the reagenic antibody (Zetterstrom and Johansson, 1981). In general, elevated levels of IgE indicate an increased probability of an IgE-mediated hypersensitivity, responsible for allergic reactions. Parasitic infestations such as hookworm, and certain clinical disorders including aspergillosis have also been demonstrated to cause high levels of IgE. Decreased levels of IgE are found in cases

of hypo-gammaglobulinemia, autoimmune disease, ulcerative colitis, hepatitis, cancer and malaria(Gibson , *et al* .,2003).The IgE serum concentration in a patient is depended on both the extent of the allergic reaction and the number of different allergens to which he is sensitized(Maiz, *et al*., 2008) who estimated the serum concentrations of immunoglobulin IgG, IgA, and IgM against *A. fumigatus* and *C. albicans* be higher in patients with cystic fibrosis than control group. Presence of *C. albicans* in respiratory secretions was correlated with the immune response to that fungus and the likelihood of obtaining *A. fumigatus* cultures from respiratory secretion increased with age.

The IgE is found on the surface membrane of basophiles and mast cells in all individuals, the IgE molecules (MW200,000), bind to the surface of the mast cells and basophilic granulocyte (Geha,1984). Subsequently the binding of allergen to cell-bound IgE causes these cells to release histamines and other vasoactive substances. The release of histamines in the body results initiates what is commonly known as an allergic reaction (Table 2).

The effect of different infectious of fungal pathogens on hematological parameters in Rabbits

The effect of *A. niger*, *C. albicans*, *Fusarium*, *A. fumigatus*, *A. flavus*, *Alternaria* inhalation in some hematological parameters including (Hb%, HCT, WBCs and differential counting) appeared significantly different in Hb% ($p \leq 0.001$) and high significant in HCT, total leucocytes, differential leucocytes was highly significant($p \leq 0.001$) (Table 3). However, Monocytes, Basiophiles were not affected during treated rabbits with fungi.

Various pathogenic effects of fungi on most of hematological parameters in comparison with control animals, Tung, *et al*., (1975).The total leucocytes count were increased while the differential leucocytic counts varied among studies with concurrent lymphopenia, monocytoses and heterophilia (Lanza, *et al*., (1980). concluded that the production of mycotoxin from pathogenic fungi caused anemia observed as decreases PCV and Hb% and increases ESR values. The values of Hb% and PCV were increased in rabbits orally treated with aflatoxin B1 (15 or 30 ng/kg body weight) every other day for nine days (Ibrahim, 2000) while using same doses they did not get affected in rabbits orally treated with 15 µg /kg body weight (Yousefa, *et al*., 2003).

Table 3: The Haematological parameters in rabbit infected with fungal infection

Rabbit No.	Type of infectious fungus	Hematological parameters in Rabbits							
		Hb (g /dl)	HCT (%)	WBC (X 10^3/mm^3)	L. (%)	H. (%)	E. (%)	M. (%)	B. (%)
1	**Control.**	12.1a±0.13	41a± 0.77	10f±0.88	61d± 0.2	37a±0.44	1c± 0.34	1a±0.27	0a±0.0
2	***A. niger***	8.9d± 0.22	27e± 0.25	20d± 0.1	80b±0.27	15d±0.10	5b±0.55	0b±0.0	0a±0.0
3	***C. albicans***	9.7c± 0.55	29d± 0.15	30b± 0.1	83a±0.13	11e± 0.09	6a±0.10	0b± 0.0	0a± 0.0
4	***Fusarium***	10.3c±0.13	31.3c±0.1	22c± 0.2	80b±0.15	15d±0.08	5±0.66	0b± 0.0	0a± 0.0
5	***A.fumigatus***	9.1d± 0.66	28d± 0.99	35a±0.19	81b± 0.1	12e± 0.12	7a±0.21	0b± 0.0	0a±0.0
6	***A. flavus***	10.3c±0.42	31c±0.33	30b±0.2	75c± 0.1	19c±0.12	6a±0.04	0b±0.0	0a±0.0
7	***Alternaria***	11b±0.75	33b±0.12	18e±0.27	74c±0.25	21b± 0.55	5b±0.13	0b± 0.0	0a±0.0

Hb=Haemoglobin; **HCT**=Hematocrit;- **WBC**=Leukocytes**, L**=Lymphocytes, **H**=Heterophils**, M=**Monocytes**, E**=Eosinophils, **B**=Basophils.

In the present study, the effect of pathogenic fungi may be due to production of crude metabolite. It was indicated that the latter had caused striking changes in the

leukocyte formula of the experimental groups although there was significant increase in the lymphocyte and mixed population (basophil, eosinophil and monocyte). This finding is in line with the report that the neutrophil percentages were gradually increased whereas the lymphocyte was decreased (Tuzcu, *et al.,* 2010). The reason for the increase of neutrophil percentage (neutrophilia) was considered due to general inflammation by intoxication and acute hemorrhage. Hemolytic anemia is a condition in which the RBC is destroyed earlier than they should be. Hemolytic anemia can cause a high neutrophil count the high eosinophil percentage (eosinophilia) is considered to be due to allergies, infections and toxin sensitivity. High basophil percentage is considered as a result of chronic hemolytic anemia and fungi infection. However, high neutrophil count can lead to leukocytosis which is associated with bacterial infection and leukemia. Low lymphocyte percentage (lymphocytopenia) is associated with mononucleosis and anemia. The low level of AFB1 has been shown to play a direct immune-suppressive effect on the cell-mediated immune (CMI) reactions by inhibiting phagocytic and microbiocidal activity of macrophages, and decreasing peripheral blood T-lymphocyte counts in the broiler chick (Oguz, *et al*., 2000). The results of the present study, however, indicate that pathogenic fungi can cause various effects on blood parameters in both human and experimental animals similar to some previous results.

Effects of inhalation of fungal conidia on IgE and IgM levels in Rabbit

The highly significant difference between the six pathogenic examined isolates, IgE titer level ranged between 130 to 425 IU/mL the isolates. The *A. fumigatus* gave the highest concentration of IgE reached to (425 IU/mL) but when treated the rabbit with *Alternaria* appeared at minimum titter (130 IU/mL) while all the pathogenic isolate appeared increased of the IgEin comparison with control. Furthermore, increasing of IgE titration, including were induced in response to *A. fumigatus* antigens in the absence of mature B cells and antibodies. (Hamelmann, *et al.,* 1997).

The significant difference between the response of IgE towered the different pathogenic fungi used seems to be related to the virulence factor of each fungi and its ability to enhance the immune system. A large titration of IgE toward *A. fumigatus* was strongly up-regulated in the presence of the metabolite of *A. fumigatus* and had raised IgE production by mucus alveolar macrophages after incubation with *A. fumigatus*. The latter was contributed to possible mechanisms illustrating the immune response during exposure to pathogenic fungi (Hohl, *et al*., 2005). Allergen-specific immunoglobulin E (IgE) plays an importantrole in eosinophil recruitment during the late-phase reaction, there for its increase during inhalationof *A. fumigates* (Yousefa, *et al*., 2003) Quantitative screening examination of IgM level in rabbi It sera treated with pathogenic isolates was found significantly variable ($p \leq 0.001$) [Table 4].

Table 4: Effects of inhalation of fungal conidia on IgE and IgM levels in blood Rabbit.

Treatment No.	Type of fungus infectious	IgE (I.U/ml)	IgM (mg/dl)
1	**Control**	27± 0.22	20f± 0.11
2	***A. niger***	305± 0.0	300d± 0.27
3	***C. albicans***	255± 0.27	312c± 0.34
4	***Fusarium***	154± 0.12	240± 0.51
5	***A. fumigatus***	425a± 0.29	390a± 0.26
6	***A. flavus***	293± 0.88	323b± 0.03
7	***Alternaria***	130± 0.13	174e± 0.44

a, f: Values within columns followed by different letters differ significantly at probability 0.001.

The IgM levels ranged between (174-390 mg/mL) which was the highest production with *A. fumigatus* and lowest production with *Alternaria.* Therefore, the hyper increase of IgM during infection of pathogenic fungi might be attributed to the induced allergy. A strong IgM response with pronounced antibody production plays a role in preventing the pathogenesis (Kurup, *et al*., 2007). These results are concomitant with other data obtained with deficient mice exposed to *A. fumigatus* (Korsgren, *et al.,* 1997; Hamelmann, *et al.,* 2003).

CONCLUSION

It is concluded that:

1. Various hematological changes appeared in the lung of both human and rabbits.
2. The immunological parameters approved that IL-4 and IgE in human meanwhile IgE,IgM in rabbits are sensitive test for detection of allergic fungi infections.

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